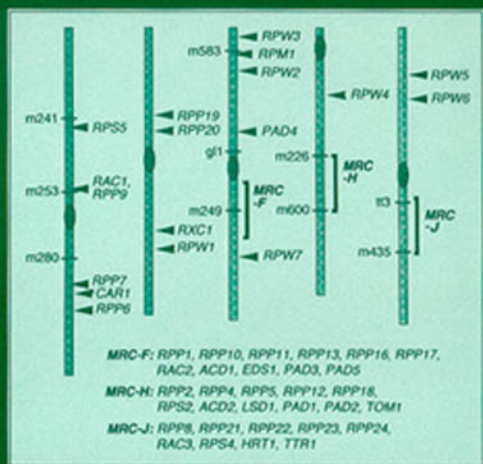




The Gene-for-Gene Relationship in Plant-Parasite Interactions

Edited by I.R. Crute, E.B. Holub and J.J. Burdon
for the British Society for Plant Pathology



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Edited for the British Society for Plant Pathology by

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Preface

This book has its origins back in 1993 when one of us (I.R.C.) accepted the nomination as Vice-president of the British Society for Plant Pathology. In the tradition of the Society, the Vice-president becomes President-elect and President in succeeding years and is accorded the pleasure of choosing the theme for the main residential meeting of the Society during his presidency. Consequently, in December 1995, the BSPP Presidential meeting addressed the theme of: 'The gene-for-gene relationship: from enigma to exploitation'.

The meeting was planned to explore what was known and unknown about gene-for-gene specificity in host-parasite interactions at the molecular, cell, plant and population levels of organization. A further emphasis was the way in which current knowledge is being exploited for control and how new insights may lead to new approaches. Recent advances in the isolation and sequencing of several genes involved in specific pathogen recognition made the meeting particularly timely and, from the outset, one intention was to provide a forum for exchange of information and ideas among the diversity of scientists with an interest in gene-for-gene relationships. For example, the efficient utilization of 'natural' resistance genes in agriculture currently requires an understanding of interactions between crop and target pathogen populations; as resistance genes are moved and utilized, as transgenes, within and between species, a similar level of understanding will be required to ensure their effective exploitation. Judged by attendance alone, the meeting was a success comprising a blend of verbal and poster presentations and a delegate list of over 200.

Because of the broadly based interest in the topic of the meeting, it was decided that a publication would be timely and place on the record the

state of knowledge as the year 2000 approaches and from which progress in the coming decades can be measured. Although all speakers at the meeting were invited to contribute to this book, there was never an intention that it would simply record the proceedings. Additionally, many excellent reviews have been written about various aspects of the gene-for-gene relationship over the last 25 years or so; no attempt is made in this book to provide a comprehensive restatement of historical findings. Rather, the intention has been, through multiple authorship of a series of chapters, to attempt a synthesis of the most exciting recent developments in understanding the gene-for-gene relationship and the practical utilization of this information.

This book addresses three themes: genetic analyses and utilization of resistance; population genetics; and cell biology and molecular genetics. The contributions within each theme have been the responsibility of a single editor whose own perspectives are presented in the form of a preamble to each of the three sections.

The gene-for-gene relationship has been a compelling and unifying force in the study of plant-parasite interactions since it was first advanced by Flor during his classical career-long studies on flax rust in North Dakota starting in the 1930s. We hope that readers will be both provoked and stimulated by the contents of this book and will sense the excitement of the authors who are all active researchers in this rapidly advancing field of enquiry.

Ian Crute
Eric Holub
Jeremy Burdon

Genetic Analyses and Utilization of Resistance

I

The elucidation of the gene-for-gene relationship and its acceptance as a framework in which to consider variation for genotype specific interactions between plants and their parasites results from many painstaking investigations of the inheritance of resistance and virulence – primarily of course, the pioneering work of H.H. Flor with flax and flax rust. Additionally, the raw material for these investigations has come, for the most part, from the practice of plant breeding for improved resistance to pests and diseases and the frequently observed lack of durability resulting from selection of virulent parasite variants.

The literature on the genetics of interactions between parasites and their hosts is legion and has been the subject of many useful and comprehensive reviews of differing flavour and perspectives. It is however possible to make a few general statements that require further elucidation:

- Plants have evolved and maintain a vast genetic repertoire allowing recognition and response to parasitic variation.
- Characteristic interaction phenotypes are associated with the operation of different recognition genes – there are degrees of compatibility.
- Genes involved in parasite recognition tend to be organized in distributed complexes or comprise multiple allelic series.

In recent times, understanding of the above phenomena has been advanced through concentration on some particularly suitable experimental systems; the exploitation of molecular markers and specially constructed mapping populations to provide high genetic resolution; recognition and elucidation of non-allelic interactions; and the identification and genetic characterization of mutants. The first three papers in this section between them provide a clear

statement of advances being made towards an understanding of the fine structure and organization of resistance genes in plant genomes, mechanisms that are involved in the evolution of specific pathogen recognition capability and the way genes at different loci interact to bring about the observed phenotypic variation.

Eric Holub describes how investigations of variation for virulence among pathogens of *Arabidopsis* has revealed many specific recognition genes and several regions of the host genome seemingly of particular importance in defence. The power of *Arabidopsis* as a non-crop model for evolutionary and ecological investigations in addition to its well-established value in plant molecular genetics is well illustrated.

By reference to several systems but primarily the *RP1* locus for rust resistance in maize, Scott Hulbert and colleagues describe the fine structure of a complex resistance locus and the mechanisms of recombination that can result in the generation of novel recognition capability. Of considerable interest is the notion of harnessing these mechanisms to produce new genes or gene combinations of particular practical utility and durability for disease control.

Mutation analysis has clearly demonstrated that the expression of resistance requires the concerted action of genes at loci other than those identified among natural variants of a host species and conceptualized as being involved as primary determinants of gene-for-gene specificity. Paul Schulze-Lefert and colleagues describe studies of non-allelic interactions between specific resistance genes and loci identified by mutation which will surely provide a fuller comprehension of the signal transduction pathways leading to resistance.

Despite what is frequently written in elementary texts of plant breeding and pathology, pathotype specific resistance has been and continues to be the mainstay of crop genetic improvement programmes with many successful applications. However, it is undoubtedly true that intensive agricultural monoculture provides a stern test of the durability for any resistance gene. Among the several approaches to enhancing the sustainable efficacy of resistance that have been suggested, the deployment of genotype mixtures is perhaps the most successful. Such an approach demands a level of knowledge of the pathosystem that may be available only for host-parasite combinations that have been intensively researched. Adrian Newton describes the gains to be made from use of cultivar mixtures, the mechanisms that might bring about these benefits and the way their use can be successfully integrated with intensive agricultural practice.

Although it is with fungal and bacterial pathosystems that gene-for-gene relationships have primarily been established, it is becoming increasingly evident that the outcome of specific interactions between plants and viruses as well as invertebrates and parasitic higher plants follow the same basic patterns and are dictated by the status of specific matching gene pairs in either partner. In addition, a remarkable and unexpected similarity has recently been demon-

strated among the products of genes from different plant species which are involved in determining the outcome of specific interactions with a diversity of microbial parasites. Systems need to be developed to determine if these same classes of plant genes will prove important in the specific recognition of invertebrate and angiosperm parasites. Athene Lane and colleagues provide an overview of resistance of plants to parasitic higher plants; in relation to gene-for-gene relationships, a study in its infancy. At the level of available knowledge, the work forcibly illustrates the need for basic information on variation for resistance and virulence together with data on genetic control. At the same time, however, the work discussed shows how it is possible now, as in the past with other systems, to make practical advances in control without a highly refined level of knowledge.

Between them, these five chapters on genetic analyses and utilization of resistance provide a brief but nevertheless embracing appraisal of the state of current knowledge and its application with optimistic views of how we can expect understanding to advance.

I.R. Crute

Organization of Resistance Genes in *Arabidopsis*

1

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We are witnessing a marriage of disciplines between natural history and molecular biology as a direct consequence of progress being made in the genetics and molecular biology of plant disease resistance. This is particularly well illustrated by efforts aimed at mapping genes in the ephemeral crucifer, *Arabidopsis thaliana* (mouse-ear cress), that are required for resistance to a wide spectrum of viruses and both microbial and invertebrate parasites. The theme of this chapter, therefore, is to examine ways in which the natural history of a common wild flower, as viewed through molecular investigation of its genome, may contribute to a greater understanding of how disease resistance has evolved in plants.

Stamp Collecting Becomes an Empirical Science

From a utilitarian perspective, the activity of mapping genes required for disease resistance in a wild species such as *Arabidopsis* will provide a genetic inventory that will aid programmes of crop improvement. Biotechnology will be advanced by broadening the gene pool from which genes can be transferred artificially across species barriers, and by unveiling opportunities for genetic engineering of novel resistance. More importantly, plant breeding will be aided by the genetic 'road map' of genes and flanking DNA sequence in the wild species that can be used to develop molecular probes for marker-assisted selection of disease resistance already existing within germ plasm of a crop species (Michelmore, 1995).

In the scientific quest to understand the molecular nature of disease resistance, gene mapping has been used successfully as a means to an end. For

genes which are known to exist only by virtue of a characteristic phenotype, the method of positional or map-based cloning has been used routinely by molecular biologists to pinpoint the location of a gene with flanking markers in an interval of DNA small enough to be carried by a transformation vector. In fact, it has been the expectation that 'anything that can be genetically mapped, can be cloned' along with application of advanced molecular techniques that largely have been responsible for establishing *Arabidopsis* as a model organism of plant biologists (Meyerowitz, 1987; Somerville, 1996). Several genes required for disease resistance have been isolated using variations of the positional cloning method including two bacterial resistance genes from *Arabidopsis* (Bent *et al.* 1994; Briggs and Johal, 1994; Mindrinos *et al.*, 1994; Grant *et al.*, 1995; Staskawicz *et al.*, 1995).

The quest to understand how disease resistance has evolved in plants and how the necessary polymorphism is maintained within a host species has been an important subject of debate (Bennetzen and Hulbert, 1992; Pryor and Ellis, 1993) together with the role of symbiosis or 'evolution by association' as a major driving force of speciation and biodiversity (Sapp, 1994; Margulis and Sagan, 1995). Empirical examination of the theories has only recently begun to be possible in plant biology from fine-scale molecular genetics and the molecular isolation of individual genes (see Beynon, Chapter 19; Hulbert *et al.*, Chapter 2; Keen, Chapter 20; and Knogge and Marie, Chapter 17 this volume). Further mutational dissection of the signal transduction pathways responsible for disease resistance will certainly continue this trend (see Dangl, Chapter 21; and Schulze-Lefert *et al.*, Chapter 3 this volume). However, to develop fully the evolution of disease resistance in plants as an empirical science, investigations must be advanced with respect to understanding the kinds of genes and biochemical pathways involved in plant defence, the numbers of genes in each functional class that exist within a genome, the organization of those genes throughout the genome, and how these genes work in concert physiologically and genetically (e.g. suppression or enhancement of recombination).

Ideally, it will be most instructive to investigate all four aspects of disease resistance (kind, number, organization, and how the genes work) in the context of a single plant species. Parallel studies in different species are certainly essential for purposes of comparison such as examining the collinearity of DNA sequence between species in those regions of each genome that have been associated with disease resistance. In any case, a systematic approach to gene mapping and DNA sequencing will provide the basic framework to assemble a more complete knowledge of the evolution of disease resistance in plants.

Arabidopsis provides one suitable biological system for empirical investigations. This wild flower is among the easiest of organisms in which to map the location of a gene on a fine scale. Detailed genetic maps based on phenotypic and several types of molecular markers have been created (reviewed by Koornneef, 1994) with the density of markers on these maps enabling researchers to position a new gene within an average distance between loci of 1.5 cM. As

described below, yet another detailed genetic map is emerging from efforts to map parasite recognition and defence-related genes. DNA sequence of the entire *Arabidopsis* genome is expected within the decade as a primary objective of an internationally coordinated programme (Somerville, 1996). A physical map of the genome will provide the necessary skeleton for the sequence information. This is being constructed from a contiguous sequence of overlapping yeast artificial chromosomes (YACs); with a given YAC carrying an insert of 100–800 kb of *Arabidopsis* DNA. The first of the five *Arabidopsis* chromosomes has already been reconstructed as a single YAC contig (Schmidt *et al.*, 1995). One approach to building up a database of DNA sequence has been via the EST (expressed sequence tags) sequencing project in which partial sequence is obtained from random cDNA clones (Höfte *et al.*, 1993; Newman *et al.*, 1994; Somerville, 1996). Partial sequences of over 20,000 expressed genes have already been produced and made available to the research community.

There are certainly limitations to what can be learned from *Arabidopsis*, but the technical power and research opportunities of this wild flower are impressive. One can imagine from the activities described above that the task of cloning a gene will be as routine as mapping its location, searching the database of *Arabidopsis* sequence to identify candidate genes in the vicinity, and testing those genes via transformation to determine which candidate is the targeted gene. Even the procedure of *Agrobacterium*-mediated transformation by vacuum infiltration has greatly enhanced the prospects of cloning a gene by overcoming the need for tissue culture (Bechtold *et al.*, 1993; Chang *et al.*, 1994). Researchers can now justify shot-gun transformation experiments involving a hundred or more candidate clones.

Ultimately, genetic and physical maps of recognition and defence-related genes in *Arabidopsis* and functional analyses of these genes will serve as a chronicle of the ways in which a wild host species has evolved in part from past encounters with parasites. Biologists in this field of research are therefore embarking, intentionally or not, on an exploration of the natural history of disease resistance in plants.

Plant Parasites as Physiological Probes

Less than a decade ago, *Homo sapiens* was widely regarded as the only organism capable of benefiting from *Arabidopsis*. Since then, researchers have described *Arabidopsis* as a host for a growing list of pathogenic opportunists that include numerous examples of prokaryotic (bacteria and mollicute) and eukaryotic (plasmodiophoromycete, oomycete, ascomycete and basidiomycete) micro-organisms, viruses and invertebrates (nematode). This topic has been reviewed by several authors in recent years (Dangl, 1993; Crute *et al.*, 1994; Sijmons *et al.*, 1994; Simon, 1994; Kunkel, 1996).

In many cases, the pathogen isolates used by researchers were collected originally from other hosts such as brassica or tomato, and were assessed for their ability to infect and colonize accessions of *Arabidopsis*. Notable exceptions include *Xanthomonas campestris* pv. *campestris* (black rot) (Tsuji and Somerville, 1992) and two obligate biotrophs common in Europe, *Peronospora parasitica* (downy mildew) and *Albugo candida* (white blister) (Koch and Slusarenko, 1990; Holub and Beynon, 1996; Holub *et al.*, 1996), which have been obtained from field collections of *Arabidopsis*. Several pathogens can be observed to affect plants grown in protected conditions under glass or in growth chambers. Common examples, particularly in plants that have reached the bolting stage, include *Erysiphe cruciferarum* (powdery mildew) and *Botrytis cinerea* (blossom, silique and stem rot). However, there are as yet no published reports in which strains of these fungi, that were originally collected from *Arabidopsis*, have been utilized in genetic analyses of disease resistance.

In keeping with the contemporary use of *Arabidopsis* as a favoured subject for laboratory investigation, there is little debate amongst practitioners about the relative merits of investigating naturally-adapted compared with non-adapted (i.e. without known history) pathogens in this model host. The pathogen isolates are in effect regarded as physiological probes for genetic polymorphism in the host, much the same as molecular probes (e.g. restriction fragment length polymorphism, RFLP) are useful tools to identify interesting or unique DNA in the genome. Standard isolates are used to screen *Arabidopsis* germ plasm in a search for clear phenotypic difference (or functional dimorphism) between a pair of host accessions. If a difference is found which can be distinguished reliably, and if the trait is simply inherited in a cross between the two accessions, then a suitable target for gene cloning has been identified. In the current mindset of researchers, the natural history of the host/parasite combination is superfluous. Nevertheless, the procedure is in theory very simple, and one which in practice could be optimized with a plant species such as *Arabidopsis* to document systematically the relative position of a large number of functionally, and perhaps evolutionarily, related genes.

Isolate collections of different parasites and pathogens are an invaluable resource for further analyses as described below. Several examples are proposed including the use of standard isolates as a bioassay for determining the specificity of a naturally polymorphic gene or mutant allele in response to infection, and for purposes of comparative biology.

Differences in Kind: Classifying the Genes Required for Disease Resistance

Natural host and parasite variation has been the fountainhead for pathology in *Arabidopsis*. Most of the host genes are expected to be somehow involved in

genotype-specific recognition of the parasite, either in producing a receptor molecule that will interact with a gene product from the parasite, or as some other naturally polymorphic component of signalling events that serve as a trigger for plant defence. Indeed, all three of the genes isolated thus far encode what appear to be receptor molecules that are similarly characterized by a nucleotide binding site and sequence domain of leucine-rich repeats (see Beynon, Chapter 19 this volume; Bent *et al.*, 1994; Mindrinos *et al.*, 1994; Grant *et al.*, 1995). Examples of parasites and locus names for the corresponding recognition genes include: *Peronospora parasitica*, RPP; *Albugo candida*, RAC; *Pseudomonas syringae*, RPS and RPM (pv. *maculicola*); *Xanthomonas campestris*, RXC; and *Erysiphe* spp., RPW (powdery mildew).

The importance of examining natural genetic variation of the host may be obvious to plant pathologists, but it contrasts markedly with most other topics of *Arabidopsis* biology in which researchers have concentrated their efforts entirely on genetic variability created by artificial mutagenesis of a few standard accessions (Landsberg *erecta*, Ler-O; Columbia, Col-O; and Wassilewskija, Ws-O). *Arabidopsis* responds well to treatments of ionizing radiation and chemical mutagens for the purpose of selecting artificial mutants; a feature which attracted many plant geneticists to *Arabidopsis* research before the burgeoning of molecular biologists in the recent decade (Rédei and Koncz, 1992). In the past two years, *Arabidopsis* pathology researchers have also been employing mutagenesis for dissecting biochemical pathways such as systemic acquired resistance (Ryals *et al.*, 1994) and programmed cell death (Jones and Dangl, 1996), which are thought in some cases to be linked functionally with the natural polymorphic genes.

Researchers have used various approaches to select artificially-induced mutations of *Arabidopsis* in a search for alterations in parasite recognition and defence-related responses. The simplest approach has been to screen populations of mutagen-treated plants with an incompatible parasite isolate and to select individuals that exhibit a shift towards susceptibility. A majority of mutations selected in this way have resulted from a change in the specific recognition gene being investigated. This has typically been verified genetically by mapping the location of the mutated gene to the same interval of close flanking molecular markers as that previously determined for a wild-type recognition gene. Such a mutant is invaluable in efforts to demonstrate that the wild-type gene has been cloned by using the mutant as the recipient for a transformation vector containing the putative gene (for example, see cloning of the RPS2 and RPM1 genes, Bent *et al.*, 1994; Mindrinos *et al.*, 1994; Grant *et al.*, 1995). Comparison of DNA sequence between the wild-type and mutant alleles provides further confirmation that the same gene is being investigated as well as determining the actual structural nature of the mutation (base pair change, deletion or rearrangement).

Mutant screening with incompatible isolates also provides a powerful means for determining whether a cluster of parasite-specific recognition genes

exists at the same locus. When more than one parasite isolate is thought to be recognized by the same host gene, a mutant screen using one isolate can be used to determine whether mutants can be selected which exhibit a shift in compatibility that is specific to that isolate. This approach has been used to distinguish between *RPP1*, *RPP10* and *RPP26* specificities on chromosome 3 in the accessions Wassilewskija and Niederzenz that otherwise have not been separated by genetic recombination (Bittner-Eddy and Holub, unpublished; Redmond, M. *et al.*, unpublished; Holub and Beynon, 1996). Alternatively, artificial mutation can reveal the dual specificity of a single host gene capable of recognizing different pathogen gene products (Grant *et al.*, 1995).

In several cases, screening with an incompatible isolate has yielded mutations in genes other than ones that are specific to the corresponding parasite genotype (Table 1.1). For example, *Col-ndr1* was selected as a shift in macroscopic symptoms towards susceptibility following inoculation with an incompatible isolate of *Pseudomonas syringae* in a search for mutants of *RPS2*, and *Ws-eds1* was selected as a shift towards profuse reproduction by an incompatible isolate of *Peronospora parasitica* in a search for mutants of *RPP1*. These mutations are to a large degree parasite non-specific: the former mutant confers susceptibility to a prokaryotic pathogen, and also exhibits a partial shift towards susceptibility to several (but not all) incompatible isolates of the eukaryote *Peronospora parasitica* (Century *et al.*, 1995); and the later mutation appears to negate the resistance conferred by known *RPP* genes from chromosomes 3 and 4 in Wassilewskija (Parker *et al.*, 1996). Interestingly, *Ws-eds1* also supports low to moderate sporulation by *P. parasitica* and *A. candida* isolates from *Brassica oleracea* and *Capsella bursa-pastoris*. Isolates of *P. parasitica* from *B. oleracea* represent the largest group tested; six isolates have now been tested, and all appear to reproduce in the same manner.

From this evidence, it would appear that wild-type *EDS1* is a parasite non-specific gene required for function of all *RPP* genes. However, several exceptions have been observed. Low sporulation of isolates from other crucifers suggests that residual downy mildew resistance can still exist in the presence of *eds1*. Most experiments have been conducted in cotyledon tissue; however, residual resistance has been observed in true *Ws-eds1* leaves with at least one *P. parasitica* isolate (*Emoy2*) from *Arabidopsis* (Parker *et al.*, 1996). Most interestingly, exceptions have been suggested from a cross between *Ws-eds1* and *Ler-0*. *Ler-0* carries at least five *RPP* genes in the *MRC-J* region of chromosome 5 (*RPP8*, *RPP21-24*), each identified by recognition of a different *Ws*-compatible isolate (see below; Holub and Beynon, 1996). From *F*₂ segregation, at least two of these genes (*RPP8* and *RPP21*) appear to confer downy mildew resistance with apparently no attenuation by the *eds1* mutation. Using a *gl3-yi* double mutant of *Ler* (flanking phenotypic markers), the *MRC-J* region from *Ler-0* currently is being backcrossed into the *Ws-eds1* background. A new homozygous combination of *eds1* from *Ws-0* with the *Ler-0* *RPP* genes from

chromosome 5 should provide a definitive test of whether *EDS1* is universally required for the expression of *RPP* genes.

Other mutants of *Arabidopsis* have been selected using methods devised to enrich for genetic alterations in downstream, defence-related genes (Table 1.1). The *Ws-nim1* mutant was selected using a compatible isolate following pretreatment and pre-incubation of the mutagenized seedlings with 2,6-dichloroisonicotinic acid (INA), a chemical which induces systemic acquired resistance to an otherwise compatible isolate in the non-treated, wild-type host. Biochemical assays have been attempted, such as the use of thin-layer chromatography to detect deficiency in phytoalexin biosynthesis (*Col-pad1-pad4*). If a defence-related gene has already been cloned, the promoter sequence of this gene can be used to construct a GUS-reporter gene for plant transformation. By producing mutagenized seed from the transformed plants, it is then possible to attempt selection of mutations in other genes that are required for activating expression of the known protein. Such an approach has already yielded mutations which exhibit non-inducible expression (e.g. *Col-npr1*) or constitutive expression (e.g. *Col-cpr1*) of the known protein. Lesion mimic mutations such as *Col-acd1*, *-acd2* and *Ws-lsd1-lsd7* were selected

Table 1.1. Mutations of *Arabidopsis thaliana* accessions Columbia (Col-0) and Wassilewskija (Ws-0) affecting non-specific changes in defence-related responses.

Wild-type accession	Locus	Mutant description	Type of screen	Reference
Col-0	<i>ndr</i>	Non-specific disease resistance	Macroscopic symptoms	Century <i>et al.</i> , 1995
	<i>pad</i>	Phytoalexin deficient	Thin-layer chromatography	Glazebrook and Ausubel, 1994
	<i>acd</i>	Accelerated cell death	Macroscopic symptoms	Greenberg and Ausubel, 1993; Greenberg <i>et al.</i> , 1994
	<i>cpr</i>	Constitutive expresser of pathogenesis-related (PR) protein	GUS-reporter gene construct	Bowling <i>et al.</i> , 1994
	<i>npr</i>	Non-expresser of PR protein	GUS-reporter gene construct	Cao <i>et al.</i>
Ws-0	<i>nim</i>	Non-inducible immunity	Parasite reproduction	Delaney <i>et al.</i> , 1995
	<i>lsd</i>	Lesions simulating disease	Macroscopic symptoms	Dietrich <i>et al.</i> , 1994; Weymann <i>et al.</i> , 1995
	<i>eds</i>	Enhanced downy mildew susceptibility	Parasite reproduction	Parker <i>et al.</i> , 1996

simply as variants that exhibited apparently spontaneous necrosis, either as discrete or as uncontrolled lesions.

With all artificially induced mutations, genetic analyses (mapping and complementation tests) should be supported with phenotypic evidence to characterize the specific nature of the mutation. Biochemical assays are now used routinely to characterize mutants of *Arabidopsis*. Examples include INA treatment to determine whether the ability to induce systemic acquired resistance has been altered and assessment of defence-related compounds such as pathogenesis-related (PR) proteins and camalexin. Differentiation between the defence responses associated with *RPS2* and *RPM1* provides an excellent illustration of comparisons that can be made among artificial mutants of *Arabidopsis* (Reuber and Ausubel, 1996; Ritter and Dangel, 1996).

The use of parasite isolates as a bioassay is also critically important. In this context, *Pseudomonas syringae* and *Peronospora parasitica* have emerged as the standard parasites for characterizing the specificity of recognition and defence-related mutations in *Arabidopsis*. For instance, in the case of *P. parasitica*, a panel of incompatible isolates can be used to determine which of the corresponding *RPP* genes are no longer fully effective in the presence of a mutation expected to confer susceptibility. This is illustrated clearly in Table 1.2 by the gross phenotypic comparison among phytoalexin-deficient mutants of Col-0 following inoculation with six incompatible isolates. When shifts to susceptibility were observed with a mutation, it often appeared to be partial. However, there were surprises such as isolate-dependent changes with Col-*pad4*; a differential response of several mutants to two isolates, *Emoy2* and *Emwa1*, derived

Table 1.2. Single and double phytoalexin-deficient (*pad*) mutations of the *Arabidopsis thaliana* accession Columbia affecting asexual reproduction by incompatible isolates of *Peronospora parasitica* (Glazebrook *et al.*, 1997).

Col-0 Line	<i>P. parasitica</i> isolate			
	<i>Gala2</i> <i>R2</i> ^a	<i>Emoy2</i> R4	<i>Emwa1</i> R4	Hiks1 R7
Wild type	N ^b	L	N	N
<i>pad1</i>	N	L	N	N
<i>pad2</i>	N	M	N	N
<i>pad3</i>	N	L	N	N
<i>pad4</i>	H	H	H	N
<i>pad1, pad2</i>	M	L	N	N
<i>pad1, pad3</i>	R	M	N	M
<i>pad2, pad3</i>	M	H	M	N

^a*RPP* locus of gene associated with specific recognition of *P. parasitica* identified in wild-type Col-0 using the named parasite isolate.

^bSporangiophore production: H = heavy (> 20 per cotyledon), M = medium (5–20), L = low (< 5 per cotyledon), R = rare sporangiophore (1–2 in < 10% of seedlings), N = none.

from the same population and thought to be recognized by the same *RPP* gene; and what appears to be a quadratic check relationship between the double mutants *Col-pad1*, *-pad3* and *Col-pad2*, *-pad3* following inoculations with *Emwa1* or *Hiks1*. These macroscopic results have been repeated in a blind experiment; and a quantitative, microscopic evaluation of the same host/parasite combinations is underway currently (Figen and Holub, unpublished).

Likewise, a panel of compatible isolates can be used to determine whether a mutation such as *cpr* confers a universal shift in disease resistance. Also, as in the case of *Ws-lsd1*, it can be informative to compare responses among inoculations with a panel of both compatible and incompatible isolates. The *lsd1* mutation was lethal following inoculation with every isolate of *P. parasitica* tested: incompatible isolates caused rapid seedling death within 24–48 h after inoculation similar to damping-off, whereas compatible isolates sporulated heavily in mutant seedlings within a week after inoculation (indistinguishable from sporulation in wild-type seedlings) but the mutant seedlings collapsed from necrosis subsequent to sporulation (Holub, unpublished; see photograph in Holub and Beynon, 1996). Interestingly, the compatible isolate *Emwa1* was unable to sporulate in true leaves of *Ws-lsd1* (Dietrich *et al.*, 1994). In addition, *Albugo candida* which is *Ws*-compatible has been the only parasite found thus far which does not induce host cell death in *Ws-lsd1* (Holub *et al.*, unpublished).

The Magic Number: Mapping Resistance Genes in *Arabidopsis*

The number of parasite recognition and defence-related genes for which a map location has been determined in *Arabidopsis* is perhaps unparalleled by what has been achieved for any other plant species. Within *Arabidopsis* research itself, no topic other than embryo-defective mutations (Jürgens, 1994; Meinke, 1994) exceeds disease resistance with respect to the extent of the genome that has in some way been implicated in the same physiological capability. All of this has been achieved in the past seven years by a dozen or so research groups, aided by substantial cooperative effort.

A global map (Fig. 1.1) summarizes the locations of parasite recognition and other defence-related genes currently known to exist in *Arabidopsis*. Most of the genes are naturally polymorphic and are therefore expected to somehow be involved in genotype specific recognition of the corresponding parasite. A single bacterial resistance gene has been identified on each of the five chromosomes (*RPM1*, *RPS2*, *RPS4*, *RPS5* and *RXC1*). Genes involved in resistance or symptom expression to viral infection have been identified on three chromosomes (*CAR1*, *TOM1*, *HRT1* and *TTR1*). Two research groups led by Shauna Somerville (Stanford University) and John Turner and Richard Oliver

(University of East Anglia) have accepted the challenge of mapping genes for powdery mildew resistance. The parasite in this case is obligately biotrophic and there is no method for long-term storage of cultures, so each group has elected to work with a single parasite isolate. Their success in mapping genes at *RPW1*–*RPW7* on four chromosomes has been achieved by the cumbersome but unavoidable task of producing a different host mapping population for nearly every gene (Adams and Somerville, 1996). *RPP* loci represent the largest group of parasite recognition genes; 26 have been named thus far on the basis of unique specificities of interaction phenotype and evidence from genetic recombination (reviewed by Holub and Beynon, 1996).

Progress in mapping *RPP* genes has largely been due to development of the *P. parasitica* collection coupled with an intensive use of recombinant inbred host lines (described in detail, Holub and Beynon, 1996). A set of recombinant inbreds is produced from a cross between two *Arabidopsis* accessions; each inbred being derived after many self-pollinated generations of single seed

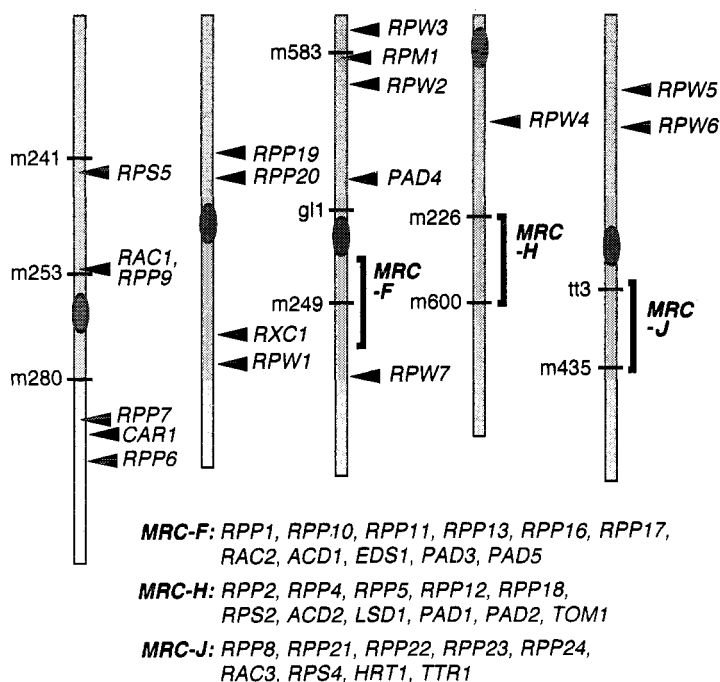


Fig. 1.1. Genetic map locations of parasite-specific recognition loci and non-specific, defence-related loci in *Arabidopsis thaliana*. Regions of approximately 20 cM that contain numerous loci have been indicated as major recognition gene complexes (MRC). Relative map positions were obtained from several sources (Holub and Beynon, 1996; Kunkel, 1996; and references listed in Table 1.1).

descent from a different F_2 individual. In *Arabidopsis*, the F_8 generation can be produced in 18–24 months if the original parents are both rapid cycling. By F_8 , most genes are in a homozygous condition, and each of the inbred lines will have inherited a different set of genes owing to recombination in a previous segregating generation. The resulting set of inbreds can then be used indefinitely for mapping purposes without the constraints imposed by segregating genes. Two sets of inbreds (F_8 Ler-0 $\times$ Col-4 and F_9 Ws-1 $\times$ Ler-W100f) were produced for general use by the research community along with an extensive database of molecular markers for each set (Reiter *et al.*, 1992; Lister and Dean, 1993). Two additional inbred sets (F_9 Col-0 $\times$ Nd-1 and F_6 Wei-1 $\times$ Ksk-1) were produced specifically for the purpose of mapping genes to *RPP* and *RAC* loci (Holub *et al.*, 1994; Holub and Beynon, 1996).

P. parasitica has proved to be highly variable and a seemingly limitless genetic resource with respect to pathogenicity in *Arabidopsis* (Table 1.3). The *Arabidopsis* gene pool alone is extensive throughout the UK (Fig. 1.2); at least one-third of UK host populations contained plants infected with *P. parasitica* (Holub *et al.*, 1994); and about one-third of the isolates obtained from a given host population represent a unique pathotype on a host differential set of 12 to 20 accessions (Table 1.3).

A genetic map of *RPP* loci that were identified in four *Arabidopsis* accessions has emerged simply by the reiterative procedure of screening inbred sets with new *P. parasitica* isolates and comparing the phenotypic data with previously acquired molecular marker and phenotypic data (Fig. 1.3). The standard host differential set used to characterize new isolates of *P. parasitica* has always included parental accessions from three of the four recombinant inbred sets (Wassilewskija, Landsberg *erecta*, Columbia and Niederzenz). Parasite isolates that exhibited differential compatibility on the parents from one of the inbred sets have subsequently been tested in the inbreds themselves. The

Table 1.3. Geographic origin and pathotypic variation of *Peronospora parasitica* isolates in *Arabidopsis thaliana* obtained from wild oospore populations of the parasite.

Geographic source	No. isolates tested	Minimum no. unique pathotypes
Canterbury, Kent	4	2
East Malling, Kent	20	6
Godmersham, Kent	4	2
Maidstone, Kent	16	4
Hilliers Arboretum, Hampshire	5	3
Aspatria, Cumbria	7	2
Edinburgh, Scotland	7	2
Ahrensburg, Germany	8	3
Wageningen, Holland	9	3
Total	80	27

phenotypic scores have typically revealed segregation among the inbreds of one or two genes from the incompatible parent. The loci can usually be mapped within a 10–15 cM interval between two molecular markers already recorded in the database of the inbred set.

The apparent clustering of *RPP* genes is an important feature of the genetic map. It is also curious that a given accession appears to have a characteristic pattern of genes. For instance, genes in Niederzenz have primarily been found on chromosome 3, and genes on chromosome 5 have thus far only been found in Landsberg *erecta*. Of course, more extensive testing of *P. parasitica* isolates is required to determine whether the pattern associated with a given accession is realistic or an artefact of sampling.

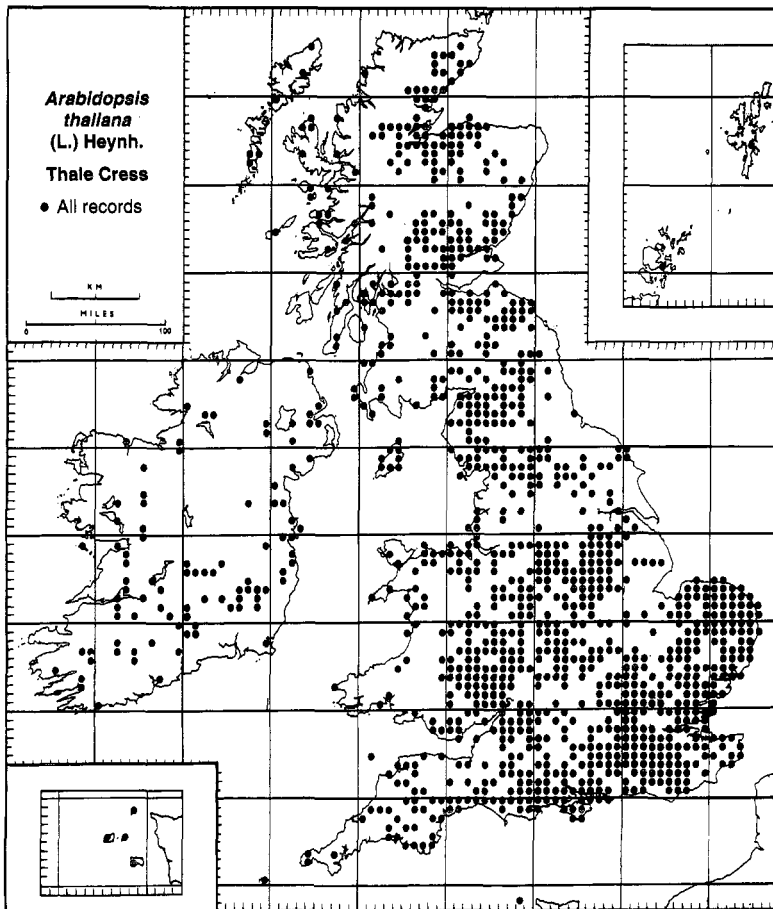


Fig. 1.2. Distribution of *Arabidopsis thaliana* in the United Kingdom (reprinted from Perring and Walters, 1962).

Organization: At Least Two Major Resistance Gene Complexes

The increasing number of *RPP* loci has made it difficult for researchers to recall where each locus maps in the genome. Major resistance gene complex (*MRC*) was therefore adopted as a convenient way of bookkeeping, providing additional information about a locus by adding an *MRC* letter that refers to a chromosome arm (Holub and Beynon, 1996). For example, *MRC-A* and *MRC-B* refer to regions on the top and bottom arms of chromosome 1, respectively. A locus designated as *RPP13* which is located on the bottom arm of chromosome 3 (*MRC-F*) can now be abbreviated as *F-13* or *RPP13f*. Most of the *MRC* regions at present are vague references to a chromosome arm because only a few genes have thus far been identified in those regions. Two regions, *MRC-F* and *MRC-J*, have none the less emerged as being of biological interest at least in part because of the growing number of genes mapping to those regions (Figs 1.1 and 1.4).

RPP genes mapping to the *MRC-F* region have been associated with the complete spectrum of interaction phenotypes including extremes in host response from flecking necrosis (only a few mesophyll cells that were penetrated by haustoria become necrotic) to expansive pitting necrosis (the parasite

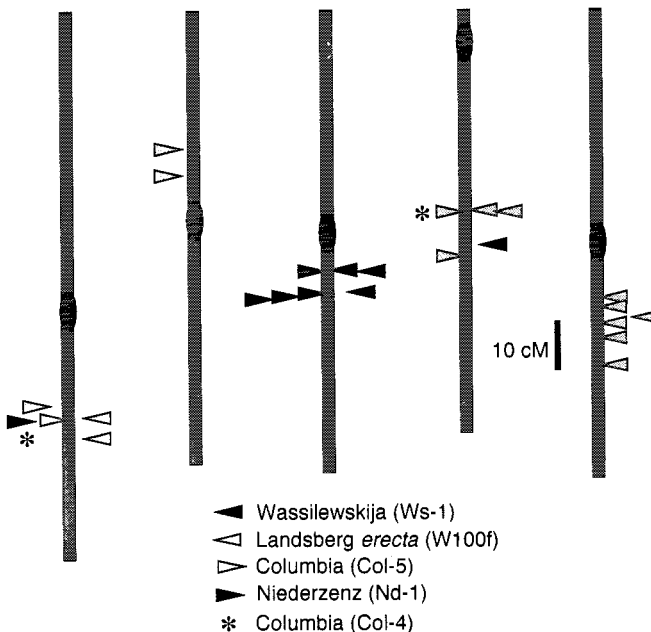


Fig. 1.3. *RPP* loci mapped in four accessions of *Arabidopsis thaliana* associated with isolate-specific recognition of *Peronospora parasitica*.

penetrates a few host cells, but the necrosis spreads further into adjacent, non-penetrated cells); and also extremes in parasite reproduction including heavy, intermediate and no sporulation (Holub *et al.*, 1994; Holub and Beynon, 1996). All of the genes shown in Fig. 1.4, except for *RPP10*, were mapped in Niederzenz using the F₉ Col-0 × Nd-1 inbreds. There appear to be at least two subclusters of loci in the regions of *RPP1* and *RPP13*. The former subcluster has thus far only been dissected by mutational analyses of the accessions Niederzenz and Wassilewskija (Bittner-Eddy and Holub, unpublished; Redmond *et al.*, unpublished). The latter subcluster has been separated on the basis of two natural recombinants (Can and Holub, unpublished), and putative mutants currently are being analysed.

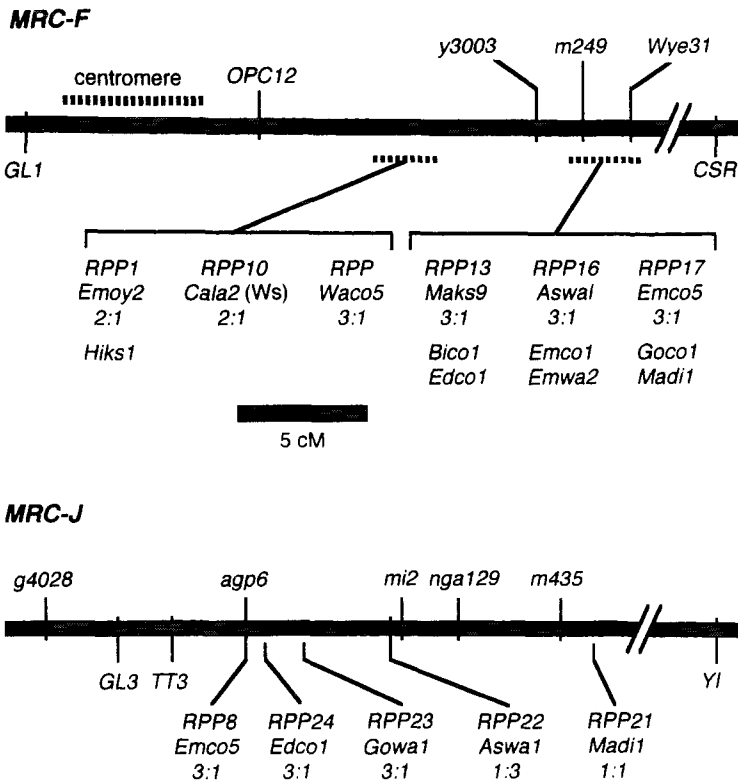


Fig. 1.4. Genetic map of major recognition complex (MRC) regions on the bottom arms of chromosomes 3 and 5 (MRC-F and MRC-J, respectively) in *Arabidopsis thaliana*. The intervals are defined by molecular and phenotypic (GL1, CSR, TT3 and YI) markers and each region contains numerous *RPP* loci associated with genotype-specific recognition of *Peronospora parasitica*. Each *RPP* locus is identified by number, the parasite isolate which was used to identify it, and an indication of its dominance at F₂ (resistant:susceptible) when crossed with a compatible accession.

RPP genes mapping to the *MRC-J* region are well defined by natural recombination unlike those in *MRC-F*. All of the *MRC-J* genes have been mapped in Landsberg *erecta* using two inbred sets, F₉ Ws-1 × Ler-W100f and F₈ Ler-0 × Col-4. Each gene is associated with a similar flecking necrosis. However, they exhibit an interesting spectrum of phenotypic dominance from *RPP8*, which segregates in a completely dominant manner, to *RPP22*, which can segregate in a recessive manner. Segregation of *RPP21* appears to be intermediate. *RPP8* is an excellent target for positional cloning because it co-segregated with the RFLP marker *agg6* in the first 100 F₈ Ler-0 × Col-4 lines tested. Unfortunately, this marker has not been released to the research community.

Both *MRC-F* and *MRC-J* appear to be suitable regions for investigating the evolution of *RPP* gene clusters. For this reason, materials are being developed which will aid future analyses. It appears to be quite easy to obtain new isolates that map a gene in Niederzenz in the *RPP13* subcluster: six new isolates are listed in Fig. 1.4 (*Bico1*, *Edco1*, etc.). These isolates will provide a useful resource for further dissection of tightly linked genes that may exist in the region. Mutational analyses will provide much of the host material which will permit distinction and relative ordering of isolate-specific genes. Natural recombinants are also critically important, so phenotypic markers which flank the *MRC* regions (glabrous loci, *gl1* and *gl3*; chlorsulphonyl urea resistance, *CSR*; transparent testa, *tt3*; and yellow fluorescence, *yi*) (Fig. 1.4) are being bred into appropriate combinations with *RPP* genes to improve greatly the selection of recombination events within an *MRC* region.

MRC regions are at present only defined genetically; however, they may eventually provide a focus for investigating the physiological and evolutionary relationships among different classes of parasite recognition and defence-related response genes. For instance, several non-specific mutations have been mapped to the *MRC-F* region including *acd1*, *eds1* and *pad3*; and several recognition genes specific to genotypes of other parasites have been mapped to the *MRC-J* region (Fig. 1.1). Clearly, researchers have only sampled a tip of the disease resistance iceberg with respect to these regions, and the genome as a whole.

Genes Working in Concert: a Genetic Approach to Reconstructing Pathways

Research that will unravel the signal transduction pathways in plants responsible for parasite recognition and defence response is the subject of several authors in this volume (see Beynon, Chapter 19; Schultze-Lefert *et al.*, Chapter 3; and Dangl, Chapter 21 this volume) and has been reviewed by others elsewhere (Innes, 1995; Kunkel, 1996; Staskawicz *et al.*, 1995). The

tremendous opportunities that can arise from mutational analyses are quite evident. Needless to say, a great deal of work remains in cloning the mutated genes and in analysing the epistatic relationships between pairs of mutations and pairwise combinations of an artificial mutation with several wild-type parasite recognition genes. Such experimentation should reveal important clues that will identify common branch points and reconstruct at least a portion of the signal transduction cascade, and variations of this theme.

None the less, the importance of natural variation still remains as scientists bring the molecular investigation of disease resistance around full circle. Having begun with examinations of naturally polymorphic host and parasite gene-pairs and then progressing to analyses involving artificial mutations, researchers will inevitably return to questions that will assess the full breadth of natural variation as the evolutionary source of disease resistance. I provide here a few examples of questions that will arise.

Do artificial mutations represent the phenocopies of natural genetic variability in a wild species such as *Arabidopsis*? In theory, any gene which can be mutated artificially has the potential of existing in nature. It therefore seems plausible that phenocopies of the mutations already selected by researchers do in fact exist somewhere at some time in nature. Although such variants may be rare compared with a major class of receptor-like molecules, they are none the less important in the evolution of signal transduction. The relative fitness of gene classes begs attention, as well as genetic propensity for change either via mutation or via recombination owing to factors such as the nature of a gene's DNA sequence, the number of gene copies, or some other structural feature of where it resides in the genome.

It is worthwhile considering whether the criteria used in the past for choosing genes as targets for molecular characterization would necessarily reveal every class of naturally polymorphic gene. Research directed specifically at finding other gene classes is required, such as determining whether genetic variation exists in expression of defence-related proteins. Efforts to isolate resistance genes could also be applied to more technically challenging examples, such as ones expressing a phenotype that is recessive, partial, temperature-dependent or dependent on genetic background.

There is a related but more specific question: can natural polymorphism be detected in more than one step of a signal transduction cascade? Mutational analyses have already revealed at least two of the steps involved in signal transduction of disease resistance (a receptor-like NBL-LRR molecule and an associated kinase molecule) (see Beynon, Chapter 19 this volume; Innes, 1995; and Staskawicz *et al.*, 1995). This would appear to contradict the gene-for-gene theory, as proposed by Innes (1995), because at least two host genes are required to make resistance possible. However, the gene-for-gene theory only refers to natural genetic variation. It will therefore remain intact until someone finds an example of two or more host components required for disease

resistance which are each naturally polymorphic in the same host/parasite interaction.

Can one recognition gene perceive more than one type of parasite? Dual specificity of a single parasite recognition gene has been substantiated by molecular isolation of the *RPM1* gene from *Arabidopsis* that recognizes two corresponding but clearly dissimilar avirulence genes from *Pseudomonas syringae* (Grant *et al.*, 1995). One could argue that this also contradicts the gene-for-gene theory; however, only one parasite gene product is required for incompatibility whether one or both of the avirulence genes are present in the parasite. The gene-for-gene basis of resistance still explains the interaction. None the less, the molecular characterization of dual specificity is of tremendous importance because it begins to explain how plants can possess more capability of parasite recognition than might be expected from the finite constraints of a genome.

The limits of dual or even multiple specificity should now be extended in a search for host genes that are capable of recognizing different parasite species. For example, the *RPS4* gene for bacterial resistance (Hinsch and Staskawicz, 1996) and the *RAC3* gene for resistance to *Albugo candida* (Borhan *et al.*, unpublished) lie in the *MRC-J* region (Fig. 1.1). By intensive screening of three parasite isolate collections, it may eventually be possible to find a host gene capable of recognizing two or more of the parasites.

Once a resistance gene has been isolated, which of its homologues are also functional as disease resistance genes, and which ones are involved in other signal transduction processes? Distinguishing between functional and apparently non-functional genes is not trivial because it depends entirely on the breadth of genetic variation in the corresponding collection of parasite isolates. The task of detecting novel specificities created from genetic recombination or rearrangement is even more daunting without diverse parasite germ plasm. None the less, both tasks are essential for investigating the evolution of a given class of disease resistance gene. The role of non-functional genes as the genetic raw material for generating new specificities will be especially interesting. In addition, DNA homology with genes from other signal transduction processes may provide clues as to the origin of disease resistance in plants. Perhaps the signal transduction that leads to disease resistance has arisen from a modification of biochemical switching in a pathway that would otherwise govern self-recognition or tissue development.

How does a plant organize genes within its genome that are somehow involved in the same physiological process? This is a fundamental question of plant biology, and disease resistance resulting from signal transduction presents an especially intriguing context for investigation. Parasite recognition genes are expected to be highly polymorphic whereas the down-stream, defence-related genes are presumably highly conserved. One could argue that this difference in allelic conservation would constrain the physical linkage of the most extreme classes of genes. In other words, how close together can the

highly polymorphic and highly conserved genes reside in the genome? A region such as *MRC-F* may already suggest that parasite recognition genes and defence-related genes can lie within a few centimorgans. The physiological link between the different classes of genes found in this region still needs to be established in detail, but this is certainly possible with mutational analyses and appropriate breeding strategies to create the gene combinations necessary for investigation. Other examples will most likely be revealed as progress is made in the international effort to sequence the entire genome of *Arabidopsis*.

How do the distributions of different parasite recognition genes (e.g. *RPP*, *RPS*, *RAC* and *RPW*) compare in the same genome? The genomic pattern of different classes of disease resistance genes is important for understanding how a given class has evolved with respect to other classes. For example, the large number of *RPP* genes reflects an important significance of this particular gene class to the evolution of *Arabidopsis*. However, it may be premature to assume that coevolution with *P. parasitica* has driven the proliferation of *RPP* genes. A comparison with the number and distribution of resistance genes currently thought to be less evolved (e.g. genes for bacterial resistance) may provide further insight. The *RPS2* gene, identified with a *Pseudomonas* isolate from tomato, itself provides an important reminder that some naturally polymorphic genes do not necessarily exist within a species as a consequence of past coevolution with a pathogen. Upon further analyses, one might predict fewer copies of such genes in *Arabidopsis*.

Genes may exist in large numbers because of their own intrinsic nature rather than as a result of coevolution. Perhaps a critical number of *RPP* gene duplications was reached which has since provided the momentum necessary for further duplication and dispersal elsewhere in the genome of that class of gene. The parasite merely influences the relative frequency of different *RPP* - alleles in the host; in such a case, stochastic events may be of greater importance in causing local extinction of a given host allele than an obligate biotroph. In this context, the role of metapopulations (see Burdon, Chapter 14 this volume) should be explored in pathosystems of *Arabidopsis*. In any case, sequence analyses of numerous *RPP* genes from throughout the genome will provide essential information in determining homologies within this gene class, patterns of distribution, and ultimately lead to speculation about how they may be evolving in *Arabidopsis*.

Concluding Remarks

In a previous essay (Holub and Beynon, 1996), an emerging trend was discussed in which researchers will begin to use comparative biology more by design than by hindsight to investigate the molecular biology and evolution of disease resistance. The importance of comparative analyses is demonstrated clearly by the tremendous advances in our understanding made possible by the

discovery that most of the resistance genes isolated thus far share similar structural domains (Staskawicz *et al.*, 1995). This was perhaps unexpected because the isolated genes are each involved in recognition of widely divergent organisms (bacteria, fungus and virus) and were obtained from several host species (*Arabidopsis*, flax, tobacco, rice and tomato). Several examples presented in the essay by Holub and Beynon (1996) provide further illustrations of the important role that comparative biology will play in future investigations of disease resistance.

We can expect a resurgence of interest in the diversity of parasites and pathogens that can infect plants. For decades, the debate about the molecular basis for genotype-specific disease resistance has been dominated by an interest in revealing the interaction between corresponding gene products from the host and the parasite. As a consequence, the pathosystems most amenable to genetic and biochemical investigation have taken centre stage. This focus of interest is clearly justified, but now that this foundation is closer to being resolved, the attention has been shifting towards investigations of downstream interactions between two host gene products. In this context, problematic organisms such as obligate biotrophs can contribute a great deal when used simply as the external stimulus for a signal transduction cascade. In *Arabidopsis*, fruitful comparisons will be possible among the cascades stimulated by the three biotrophs *Erysiphe* spp., *P. parasitica* and *A. candida* to determine whether specialization occurs after parasite recognition at the level of host response.

With recent progress in our understanding of the molecular basis of disease resistance and with increased use of comparative analyses, plant pathology increasingly will be transformed into an important facet of evolutionary biology. The most relevant questions will always address the behaviour of crop species in response to parasites and pathogens. However, *Arabidopsis* will also provide useful information, particularly where it enables the synthesis of disparate information from crop pathosystems. Greater appreciation of its wildness presents further opportunities, at the very least from the reservoir of naturally polymorphic genes still available within the species.

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Genetic Fine Structure of Resistance Loci

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A major class of resistance genes in plants is involved in recognition of specific strains of pathogens (pathotypes) in a gene-for-gene manner. Such genes may represent major fitness components in natural populations of plants which are challenged by highly variable pathogens. The value of any given gene, however, depends upon the frequency of occurrence of the corresponding avirulence gene in the pathogen population. The propensity of pathogen populations to generate virulent pathotypes is well documented in agricultural situations (see Part II of this volume). Pathogen populations that are completely avirulent owing to the presence of a specific resistance gene in the plant cannot survive unless they acquire virulence as a result of mutation and loss of the avirulence gene functions. It follows, therefore, that plant species could benefit from mechanisms that promote rapid evolution of resistance genes.

The evolutionary potential of resistance genes is reflected in their genetic structure and arrangement in plant genomes; resistance genes involved in gene-for-gene systems commonly map to specific areas of plant genomes. These areas where multiple resistance specificities map represent either clusters of linked genes or allelic series at simple loci (Shepherd and Mayo, 1972). Differentiating these two possibilities is sometimes difficult, but can have important implications for how these genes evolve and how they can be manipulated for experimental purposes or in breeding programmes. Recombination will sometimes differentiate the two cases, since genes which map several centimorgans apart are clearly not allelic. There are several examples of gene clusters where at least some of the members are relatively loosely linked (e.g. 5 cM or more). Two clusters of genes occur in lettuce which carry *Dm*

genes for resistance to *Bremia lactucae* as well as genes for resistance to several other pathogens (Kesseli *et al.*, 1994; Witsenboer *et al.*, 1995). A cluster of crown rust resistance genes has been identified in diploid oats (Gregory and Wise, 1994), and in barley there is a cluster of powdery mildew resistance genes in the *Ml-a/Ml-k* region (Giese, 1981; Jørgensen, 1992). However, very tightly linked genes are more difficult to distinguish from allelic series because the low frequencies of recombination can be confused with intragenic recombination events, especially since studies of intragenic recombination at several plant genes (Nelson, 1962; Dooner, 1986) have indicated that frequencies equivalent to 0.1 cM are not uncommon. Shepherd and Mayo (1972) designed a genetic test to discriminate between the two situations. The ability to recover susceptible recombinants and those with both parental resistance genes linked in *cis* from a heterozygote in which the two specificities were present in *trans*, indicates multiple genes. Alternatively, the inability to construct a haplotype with both parental specificities indicates allelism. An underlying assumption was that both parental specificities could not be expressed from a single resistance gene product at a simple locus, an assumption that has not yet been disproved. Shepherd and Mayo accurately predicted the structures of the *L* and *M* rust resistance loci of flax using this criterion. Recent molecular analysis has verified a simple genetic structure for the *L* locus and a gene cluster or complex locus at *M* (Ellis *et al.*, 1995).

Recombination Events in Complex Resistance Genes

While recombination has frequently been proposed as a mechanism of diversity generation at resistance loci, few studies have been conducted to test its role. One hindrance in the past has been the lack of easily utilizable markers flanking resistance genes that can be used to assay crossing-over. Another obstacle in self-pollinated plants is the low efficiency of detecting recombination or spontaneous mutation events at dominant resistance genes when large test cross populations cannot be constructed. The maize/rust interaction has been an ideal system for studies of diversity generation because of the simplicity of the resistance assay, the availability of markers, the easily manipulated genetics of maize, the extensive collection of rust pathotypes and the range of resistance phenotypes at the *Rp1* locus.

Analysis of the *Rp1-complex* of maize (for resistance to common rust, *Puccinia sorghi*) has indicated that at least some resistance gene clusters do not behave like groups of independent, linked genes in terms of how they recombine. Standard genetic terminology such as 'allele' and 'locus' are difficult to apply to *Rp1* so the term 'complex locus' has been used. This is because genes at different positions in the complex are able to pair and recombine as if sometimes they are either distal or proximal to each other. The first indication of this was the genetic instability of most *Rp1* homozygotes (Pryor, 1987; Bennetzen

et al., 1988). The mechanism of this instability was demonstrated by constructing *Rp1* homozygotes with heterozygous flanking markers. The susceptible derivatives were nearly always associated with crossing-over, but both possible non-parental combinations of flanking markers were observed in roughly equal frequencies (Sudupak *et al.*, 1993; Fig. 2.1). Differences in meiotic pairing can also be observed in *Rp1* heterozygotes, such as a *Rp1-J/Rp1-F* heterozygote. Again, susceptible recombinants (with neither gene) are associated with both non-parental combinations of markers flanking the complex locus, differentiating this from normal crossing-over between linked genes. Recombinants with both *Rp1-J* and *Rp1-F* were also selected and these too exhibited both combinations of flanking markers (Hulbert *et al.*, 1993). Although genes like *Rp1-J* and *Rp1-F* recombine fairly frequently by crossing-over, their relative

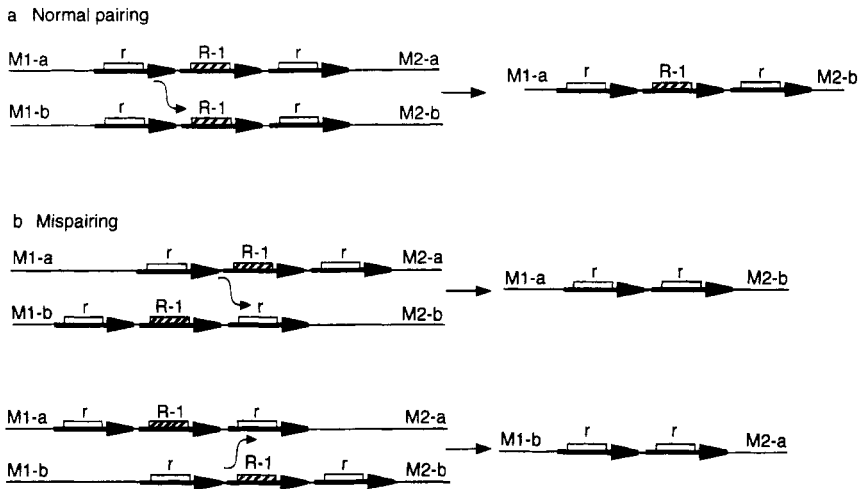


Fig. 2.1. Model for unequal crossing-over at a complex resistance gene. Lines represent regions of two paired chromosomes in meiosis. The chromosomal regions were constructed to include a complex locus which is homozygous and two flanking markers, M1 and M2, which are heterozygous. The complex locus carries a sequence which is duplicated three times (heavy arrows) and carries three members of a resistance gene family (boxes), only one of which (hatched box) is detectable using the pathogen pathotype employed for analysis. Crossing-over (bent arrow), either within the resistance genes or somewhere else on the sequence duplications, while they are misaligned, can generate gametes which do not carry the detectable resistance gene and result in susceptible progeny. (a) Crossing-over while the genes are paired equally results in no loss of sequences and no phenotypic change. (b) Crossing-over following two different types of mispairing can be observed as susceptible progeny with two different non-parental combinations of flanking marker alleles.

map position is ambiguous because they sometimes recombine as though *Rp1-J* maps distal to *Rp1-F* and sometimes as though *Rp1-F* maps distal to *Rp1-J*. Other genes appear to map more proximally or distally in the array; for example *Rp1-D* and *Rp1-A* usually recombine with *Rp1-J* or *Rp1-F* as though they map distally. Susceptible cross-over derivatives arise from *Rp1-J* homozygotes at about the same frequency as they do in heterozygous combinations of *Rp1-J* with *Rp1-F*, *Rp1-D* or *Rp1-A*, indicating mispairing probably occurs as commonly as normal pairing.

The term unequal crossing-over (UCO) is generally used to describe mispairing and crossing-over in homozygotes. The term is also used to describe mispairing in heterozygotes, but is not entirely appropriate because of the ambiguity in defining 'equal' pairing between two haplotypes that may be structurally very different. UCO, in general, requires sequence duplications which retain sufficient homology to mispair and recombine. The duplications need not be tandem (Jackson and Fink, 1985; Maloney and Fogel, 1987) and they can be as small as a single gene (or even a truncated gene, Robbins *et al.*, 1991) or large enough to be cytologically visible, like the *barr* locus of *Drosophila*. When a large duplication is mispaired during meiosis, a cross-over anywhere on the duplicated segment will generate an UCO event; the cross-over does not have to occur in the gene of interest. Thus, the very high frequency of UCO exhibited by *RpG* (6×10^{-3} ; Sudupak *et al.*, 1993), which maps 2 cM distal to the *Rp1* complex, does not necessarily indicate an unusually high rate of intragenic recombination. Rather, it may indicate the gene resides on a larger duplication than the genes in the *Rp1* complex. The generation of *Rp1* genes with novel specificities is evidence for intragenic recombination, however, and occurs frequently in some crosses (see below).

Rp1 variants have also been identified which are not associated with crossing-over and are thought to result from gene conversion events. Although it is not possible to formally demonstrate gene conversion without recovering multiple products from a single meiosis, two lines of evidence implicate conversion events (Hu and Hulbert, 1994). Non-cross-over (NCO) events were found mainly in crosses between *Rp1* genes with the highest levels of cross-over associated instability, such as *Rp1-C*, *Rp1-J* and *Rp1-F*, indicating the mechanism was probably also recombination associated. In addition, double-resistant derivatives (with both parental genes) were roughly as frequent as susceptible derivatives. Susceptible derivatives from an *Rp1* heterozygote could conceivably arise from a variety of mechanisms in addition to gene conversion, such as point mutation or insertional inactivation of one of the parental genes, or by intrachromosomal recombination. Experiments with *RpG* have indicated that intrachromosomal recombination is not involved in the high levels of instability observed at this locus; *RpG* hemizygotes (paired with an *Rp1*-area deletion) are meiotically stable. Furthermore, the generation of a double-resistant derivative from a heterozygote (e.g. *Rp1-J/Rp1-F*) by intrachromosomal recombination would not be possible. The generation of the double

resistant type by mutation would also be very unlikely. It would require one of the undetectable or 'silent' *rp* sequences in one of the parental haplotypes (e.g. *Rp1-J*) to be mutated to a functional gene with the specificity of the other parent (e.g. *Rp1-F*). Such a mutation of a silent gene to one with a known specificity has not yet been observed. A model where a silent *rp* copy is converted by a detectable gene from the other parent is more consistent with the results.

In certain other biological systems where the generation of new diversity at a particular locus or class of genes is necessary, specialized recombination systems have evolved. An example is the mitotic events which generate immunoglobulin diversity in animals. No evidence for such a specialized mechanism has been observed in recombination at the *Rp1* locus; recombination is frequent, but the types of recombination events observed are not unusual for duplicated sequences. The events observed have been predominantly meiotic, not mitotic. Crossing-over events occur mainly between chromosomes, as opposed to between duplications on sister chromatids or the same chromatid; similar observations have been made between tightly linked repeats in yeast (Klein, 1984; Jackson and Fink, 1985; Maloney and Fogel, 1987). The high frequencies of gene conversion shown by some *Rp1* genes are not unexpected for genes showing high frequencies of intragenic crossing-over since the two events occur from a common intermediate in most current models of how recombination occurs in eukaryotes (Orr-Weaver and Szostak, 1985; Nicolas and Petes, 1994). The frequent mispairing between duplications carrying the *Rp1* genes is also not uncommon; mispairing in tandem arrays has been estimated to be nearly as common as normal pairing in tandem arrays in a number of systems where duplications have been studied (Dooner and Kermicle, 1971; Maloney and Fogel, 1987).

Crossing-over and gene conversion probably play a similar role in diversity generation at simple resistance loci with multiple alleles as that at complex loci. However, in the case of simple loci, the number of variant forms of a gene which can pair and recombine will be more limited. This is particularly true of self-fertilized plants whose populations are composed mainly of homozygous individuals, or of any plant populations with limited genetic variation at the locus. The ability of complex loci to carry two or more alleles in a single haplotype can preserve this variation even in small inbred populations and thus preserve the potential for recombination between alleles.

It is not known how commonly other resistance gene clusters recombine as though they are complex loci, like *Rp1*. Factors that will effect the occurrence and frequency of mispairing and recombination are the distance between the repeats on which the genes are carried (Hipeau-Jacquotte *et al.*, 1989) and the degree of sequence divergence between the repeats (Wheeler *et al.*, 1990; Metzenburg *et al.*, 1991). The orientation of the repeats with respect to each other will also affect the recovery of recombinants since interchromosomal cross-overs between inverted repeats create acentric and dicentric chromosomes (Petes and Hill, 1988). These factors will depend in part upon how long

ago the duplications formed and the mechanism by which this occurred. The manner in which such gene clusters are formed is not known, but it is often assumed to be the result of duplication of genomic segments carrying the genes. An initial tandem duplication could be created by a rare ectopic crossing-over event (between homologous sequences at non-homologous locations) between linked repetitive elements. Such recombination events have been demonstrated between *roo* elements in *Drosophila* (Montgomery *et al.*, 1991) and *Alu* repeats in humans (Meuth, 1989). The size of the duplication would then depend on the distance between the repeats. It is possible then that the more loosely linked resistance genes resulted from very large duplications. Duplications that formed long ago may be unrecognizable owing to sequence divergence and localized rearrangements, and only certain genes may be sufficiently conserved to retain synaptic homology. Regardless of the mechanism by which they were formed, any genes which exist between the resistance genes whose deletion or dosage imbalance are deleterious should make UCO events difficult to recover.

During genetic analysis of a resistance locus, there are a number of factors to look for that might be indicative of UCO. One is loss of resistance in the progeny of homozygotes, although not all instability in gene families will be associated with crossing-over (Walker *et al.*, 1995); demonstration of crossing-over may require the breeding of heterozygous flanking markers into the resistance gene homozygote. Observation of two different types of crossing-over in susceptible progeny from a line that is heterozygous at the resistance gene is also good evidence of UCO and flanking DNA markers are more likely to be assayable. It may be more informative to test a number of different crosses than to concentrate on just one; the frequency of UCO events at *Rp1* varies widely with the allele.

Obtaining the sequence of a resistance gene opens new possibilities for genetic analysis of complex loci. Several (Martin *et al.*, 1993; Jones *et al.*, 1994; Whitham *et al.*, 1994; Ellis *et al.*, 1995; Loh and Martin, 1995; Song *et al.*, 1995; Dixon *et al.*, 1996), but not all (Johal and Briggs, 1992; Bent *et al.*, 1994; Mindrinos *et al.*, 1994; Grant *et al.*, 1995) of the resistance genes which have been cloned hybridize to several genetically linked DNA fragments in gel blot analysis of genomic DNA, indicating a complex locus or gene cluster. Since UCO events change the numbers of copies of the duplicated sequence each time they occur, the use of the cloned gene as a probe to assay copy number will be a powerful tool to analyse recombination events. A first indication of UCO would be if different plant lines carry different numbers of copies of the duplication (Hong *et al.*, 1993). A more direct approach would be to look for changes in homologous fragment numbers in progeny showing a loss of resistance or change of specificity. Thus, a rare recombinant between the tomato *Cf2* and *Cf5* genes showed a reduced number of sequences homologous to the *Cf2* gene as compared with the parents (Dixon *et al.*, 1996). Recombinants are more readily obtained between *Cf4* and *Cf9* and an ongoing analysis of these

variants will indicate if multiple types of meiotic pairing and recombination occur at this locus (J.D.G. Jones, personal communication). In complex loci composed of non-tandem duplications there may be unique sequences between the resistance genes, and loss or duplication of these sequences would also indicate unequal exchange. An interesting aspect of the recently cloned *RPM1* gene of *Arabidopsis* (Grant *et al.*, 1995) is that the sequence is entirely missing in strains which are susceptible to *P. syringae* expressing *avrRpm1*, possibly owing to loss associated with some type of genomic instability.

Generation of Novel Specificities by Recombination

Most recombination analyses of resistance genes are set up to look for susceptible types from F₁ hybrids that are heterozygous at the locus and use a single pathotype to screen the progeny. Some analyses have also used two complementary pathotypes to try to select 'double-resistant' recombinants with both parental alleles linked in coupling (Saxena and Hooker, 1968; Shepherd and Mayo, 1972). In a few studies, most notably the maize and flax rust systems, investigators have looked for, and found, variants that were neither completely susceptible nor double-resistant types, but exhibit novel resistance specificities. In at least some cases, these variants appear to be novel alleles, but the generation of a novel specificity does not necessarily indicate the creation of a novel allele, especially when considering complex loci or gene clusters. Here reassortment of existing genes in the parents could create a haplotype with a novel specificity that is difficult to distinguish phenotypically from an actual novel gene. This can occur when one or both of the parents actually carry two **detectable** genes: while most lines may carry several functional resistance genes at a complex locus, the only detectable genes are those for which an avirulent pathogen pathotype is available to assay its presence, other alleles are silent. When two such genes are separated by recombination, a non-parental specificity is generated. The recombinant will be resistant to a subset of the pathotypes that the parent with two genes is resistant to if it did not receive a detectable resistance gene from the other parent (Fig. 2.2a). If it receives genes from both parents, like the reciprocal product of the recombinant in Fig. 2.2a, its resistance may not be a subset of resistances of either parent, but it will be resistant to a subset of the pathotypes that the parental hybrid was resistant to. In either case, the variants that arose by reassortment of existing genes should not be resistant to any pathotypes that neither parent was resistant to. In contrast, a novel resistance gene may be resistant to some pathotypes that neither parent was resistant to.

Thirteen lines with altered resistance specificities were identified at the *Rp1* complex by examining approximately 200 lines derived from recombination events in the *Rp1* area with eleven different rust pathotypes (Richter *et al.*, 1995). Most of the recombinants examined were selected for the absence of

either parental gene from *Rp1* heterozygotes, and all but a few were found to be susceptible to all rust pathotypes selected. Most of the variants with altered specificities were originally selected on the basis of a modified resistance phenotype that appeared different from either of the parents after inoculation with the rust pathotype they were originally screened with. Of eleven variants selected with modified resistance, all but one showed unique resistance specificities to the collection of rust isolates; the other simply showed reduced levels of resistance to all rust pathotypes that the *Rp1*-D parent is resistant to. Selection

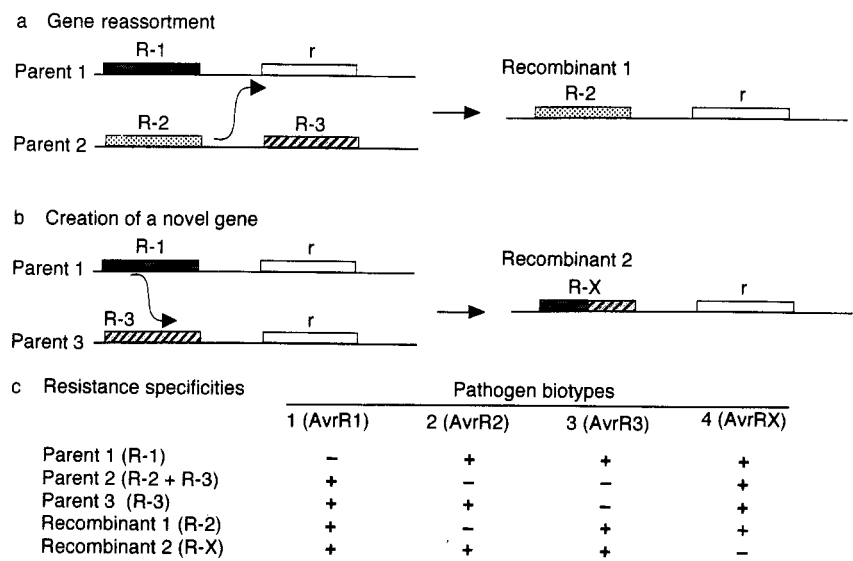


Fig. 2.2. Models for the creation of novel specificities at complex resistance genes by cross-over events.

(a) Reassortment of existing genes. Lines represent the resistance gene complex from two different parental lines, each carrying two different resistance genes (boxes). An F_1 hybrid between parent 1 and parent 2 carries three resistance genes that are detectable with the four available pathogen pathotypes. Meiotic crossing-over (arrow) in the F_1 , in a region of homology in the complex separate the two detectable genes from parent 2, creating recombinant 1 which carries a novel combination of genes.

(b) Creation of a gene with a novel specificity. Intragenic crossing-over between two genes creates a resistance gene (*R-X*) capable of recognizing a different avirulence gene in the pathogen (*AvrRX*).

(c) Reaction of the parental lines and the recombinants with four hypothetical pathogen pathotypes (– = incompatible interaction; + = compatible interaction). Recombinant 1 has a different specificity to that of either parent, but is not resistant to any pathotypes that neither parent was resistant to. Recombinant 2 is resistant to one pathotype (4) to which both of its parents were susceptible.

for modified resistance phenotypes was, therefore, an efficient method for identifying altered specificities.

Four of the *Rp1* variants with altered specificities were resistant to at least one rust pathotype that both parents were susceptible to and were considered to represent novel resistance genes. All four were associated with cross-over events. The other nine variants had a different specificity than either parent, but were not resistant to any pathotypes that both parents were susceptible to. It is not known, therefore, if these represent the creation of genes with novel specificities (Fig. 2.2b) or reassortment of genes in the parents (Fig. 2.2a). The main difficulty in interpretation of these events comes from not knowing if any of the parental specificities are controlled by two different detectable genes. The existence of two detectable *Rp* genes linked in *cis* has never been demonstrated in any maize lines, but recombinants with two or even three *Rp1* genes in a single haplotype have been constructed experimentally. Similarly, no two detectable *M* genes have ever been demonstrated conclusively at the *M* locus in flax, except for those constructed experimentally. Linkages in coupling have been identified, however, in resistance gene clusters where the members are more loosely linked, such as *Dm* genes of lettuce (Hulbert and Michelmore, 1985), the *Pca* locus of oats (Gregory and Wise, 1994), and the *MI-a* – *MI-k* region of barley (Giese, 1981; Jørgensen, 1992).

Variants at the *L* locus of flax with modified resistance phenotypes have been identified from a number of heterozygotes (Islam and Shepherd, 1991a, 1991b). In some cases, such as *Lx* selected from an *L2/L6* heterozygote, these represent novel resistance specificities (Lawrence *et al.*, 1994). It is not known if the *Lx* allele arose by a cross-over event because flanking markers were not available for analysis. The *Lx* specificity did not show resistance to any rust pathotypes that both parents were susceptible to, as some of the *Rp1* recombinants did. It seems likely, however, that *Lx* represents a novel resistance gene because the simple structure of the *L* locus (Ellis *et al.*, 1995) precludes reassortment of parental genes as an explanation; each of the parents should have only a single allele at the *L* locus. In addition, there is genetic evidence from segregation analysis of avirulence in the pathogen that the *Lx* specificity detects a different *Avr* gene than either of the parental genes, indicating *Lx* is essentially a novel resistance gene (Lawrence *et al.*, 1994). This latter observation demonstrates the utility of genetic analysis of the pathogen in examining altered specificities in the host.

An interesting aspect of the specificity changes observed at the *Rp1* and *L* loci is the number of different specificities that have been generated from a single cross. If it were possible to generate a very large number of different specificities by recombination or mutation in a given heterozygote, then one would expect different derivatives from a single cross to all have different specificities. That has not been the case in the analyses to date. Of five derivatives with modified resistance that were selected from an *L2/L10* hybrid, all appeared identical in specificity. The single maize cross in which the most altered

specificities were identified was an *Rp1-K/rp1* heterozygote, where five variants were isolated from only 2700 progeny. These fell into three different specificity classes, two pairs of recombinants which had identical specificities with each of eleven different rust pathotypes and one recombinant with a unique phenotype. At least two, possibly all three, of these classes were thought to be novel genes because of their resistance to a rust pathotype to which *Rp1-K* was susceptible. It seems, therefore, that there is either a finite number of specificities that can be easily generated by recombination from a given gene or pair of genes, or a finite number that can be detected with a given collection of pathotypes.

Chemical mutagenesis experiments of barley lines carrying the *Mla-12* resistance gene resulted in 25 variants with reduced resistance to powdery mildew (Jørgensen, 1987). Of these, 22 of the mutants mapped to the *Mla-12* locus. None of the mutants were completely susceptible to powdery mildew and none showed an altered specificity when tested with several mildew pathotypes. The results of this experiment were, therefore, quite different than those of any of the genetic analyses of *Rp1*. Recombination at *Rp1* commonly gives individuals with no detectable resistance, and variants with reduced resistance have usually had an altered specificity when examined with multiple pathotypes (Richter *et al.*, 1995). Only one variant has been identified, *Rp1-D5*, that has a reduced resistance but no apparent change in pathotype specificity. A number of possibilities could explain the difference between the results of *Mla-12* and *Rp1* experiments. One possibility is that the *Mla-12* locus is composed of two or more genes with identical specificities. This was recently found to be the case for the *Cf2* locus of tomato (Dixon *et al.*, 1996). Other possible reasons for these differences stem from the technical differences in the way the experiments were conducted. Most of the *Rp1* variants have been generated by spontaneous recombination events. The *Rp1-D5* variant was exceptional in this regard in that it is probably the result of transposon mutagenesis (Pryor, 1993). Another technical difference is that the *Rp1* variants have mainly been identified in outcross progeny; generally from a resistant homozygote or F_1 crossed to a susceptible (tester) line. In contrast, the *Mla-12* variants were identified in the self-fertilized progeny of mutagenized homozygotes. If some complex loci are composed of two (or more) different classes of genes, one of which controls pathotype specificity and another required for full expression of resistance, then mutants derived from self-fertilization might be more likely to identify mutants in the second class of genes. Identifying these mutants in test cross progenies would require that the tester parent carried the recessive allele at the 'expression' locus as opposed to the locus controlling pathotype specificity. Experiments to identify *Rp1* variants from self-fertilized progeny of mutagenized *Rp1-D* homozygotes are currently underway to determine if different classes of genes are identified than those identified in the *Rp1* recombination experiments.

Efficient Detection of Resistance Gene Variants

Molecular analyses of resistance gene variants with altered phenotypes will tell us much about their evolution, their mode of action and how they might be engineered *in vitro*. Experiments with *Rp1* have provided some indications of how to identify these variants efficiently. Nearly all of the *Rp1* variants have been selected following inoculation of an *Rp1* homozygote or heterozygote with a single rust pathotype and selecting for altered phenotypes. Variants that appear completely susceptible to the pathotype used in the original screen have been, for the most part, susceptible to all pathotypes. Variants that were selected for a modified resistance phenotype have been more interesting in that they have usually acquired a novel specificity when their progeny were subsequently tested with other rust pathotypes. Individuals with altered resistance phenotypes were most easily identified from parents whose resistance genes are associated with very consistent phenotypes, such as those that show complete resistance (no sporulation) regardless of greenhouse conditions. Several of the *Rp1* genes which show intermediate resistance are also less consistent in their expression, and individuals selected as phenotypically different often show the parental phenotype when progeny tested.

The frequencies of recombination events leading to novel *Rp1* genes vary considerably between crosses and the most 'productive' crosses for finding novel types are not predictable. The highest frequency of novel *Rp1* variants came from a specific *Rp1-K/rp1* heterozygote: *Rp1-K* was previously thought to be one of, if not the most stable and least recombinogenic of *Rp1* genes both in homozygotes (Bennetzen *et al.*, 1988) and heterozygotes (Hulbert and Bennetzen, 1991). Identification of an *Rp1-K* heterozygote that recombined frequently, indicated the gene could be very recombinogenic but that it depended upon what it was paired with in meiosis. It is also clear from this cross, and others, that it is not necessary for both parents to carry detectable resistance genes to generate novel types by crossing-over. Apparently the silent genes carried in susceptible lines can contribute useful genetic information. In view of the unpredictability of which crosses will be productive in generating novel types, probably the best way to ensure a productive cross is to try a number of different crosses. Also unpredictable, is the best pathotype for identifying novel variants from a given cross. All of the *Rp1* variants with novel race specificities might not have been identified if a different rust pathotype had been used in the original screen; with a different pathotype, they might have appeared phenotypically identical to the parental gene or possibly completely susceptible. It is also possible, however, that if different rust pathotypes were used, a different set of variants would have been isolated. Other types of recombinants, however, discussed below, can be identified regardless of the pathogen pathotype used for screening and are sometimes detectable even in the absence of the pathogen.

Variants with Pathotype Non-Specific Effects

Rp1 variants have been identified which react in a similar manner to all rust pathotypes tested, regardless of their reaction to the parental *Rp1* genes. The first of these identified was found as a progeny seedling from an outcross of an *Rp1-D* homozygote that showed a highly necrotic reaction to an Australian rust pathotype which was avirulent on *Rp1-D*. The parental *Rp1-D* phenotype is highly resistant with no visible necrosis and the hypersensitive reaction is confined to very small patches of cells. The highly necrotic reaction of the variant gene, called *Rp1-D21*, differs from the *Rp1-D* reaction both in the extent of cell death and also in allowing some colonization and sporulation to occur. When challenged with nine different rust pathotypes from a *P. sorghi* collection, lines carrying the *Rp1-D21* gene always reacted in an identical manner regardless of whether the pathotypes were virulent or avirulent on the parental *Rp1-D* gene. This indicated that the specificity of the *Rp1-D* parent was lost in the event which gave rise to the variant. Since the *Rp1-D21* gene arose from an *Rp1-D* homozygote, RFLP markers flanking the *Rp1* complex were also homozygous and it was not therefore possible to determine if it was generated from a cross-over event.

A second *Rp1* variant with a similar phenotype, *Rp1-NC3*, was identified among progeny of a *Rp1-C/Rp1-N* $\times$ *rp1/rp1* (H95) cross. Analysis of the flanking markers indicated it arose from a cross-over event in the *Rp1* complex. Like *Rp1-D21*, *Rp1-NC3* reacts identically to all rust pathotypes tested, indicating the loss of parental specificity and the production of a new, apparently pathotype non-specific, reaction type. The necrotic reactions of both *Rp1-D21* and *Rp1-NC3* after inoculation with rust appear similar to the hypersensitive response (HR) associated with other *Rp1* genes following histological examination with several stains that detect compounds (e.g. callose) generally associated with HR. The necrotic reactions are also associated with a significant reduction in sporulation of the fungus, but colonization and pustule formation are not completely prevented.

The pathotype non-specific nature of the *Rp1-D21* and *Rp1-NC3* alleles is illustrated further by inoculation with other rust species. Lines carrying either gene exhibit the characteristic rapid necrotic reaction when inoculated with *P. polysora*, the maize 'southern rust' pathogen. Lines carrying these genes also react strongly to species of rust from other hosts which are not pathogens of maize, such as *P. recondita*, the wheat leaf rust pathogen.

Another interesting aspect of the *Rp1-D21* and *Rp1-NC3* phenotypes, is that both are associated with necrotic spotting in the absence of any rust. Both lines exhibit classic 'disease lesion mimic' phenotypes (Walbot *et al.*, 1983) when grown either in the field or the greenhouse. Like many maize lesion mimics mutants, the spots are generally not noticeable until the leaves are fully expanded. Some necrotic spots are usually observable on leaves of *Rp1-D21* seedlings, but the number of spots generally increases as the plant matures and

the phenotype has a negative effect on overall fitness. This is particularly true of *Rp1-D21* homozygotes, which generally do not set seed in the field. The *Rp1-NC3* phenotype is generally less severe. It is often not noticeable until the adult plant stage, and is somewhat dependent upon genetic background and environmental conditions such as temperature. Furthermore, the *Rp1-NC3* phenotype is only expressed well in homozygotes and is usually not noticeable in heterozygotes.

When seeds of lines carrying *Rp1-D21* and *Rp1-NC3* were surface sterilized and grown aseptically in sterile media, the lesion-mimic phenotypes were not observed. This indicates the necrotic reactions do not occur 'spontaneously' and that a biotic stimulus is required for the expression. The pathotype specificity of the *Rp1* genes in the parents of *Rp1-D21* and *Rp1-NC3* were lost in the events which gave rise to these variant alleles. If these variant alleles are altered in a ligand-binding type of recognition domain, they may either recognize a broad array of pathogen associated compounds or possibly a metabolite that is commonly made in the interactions between plant cells with a variety of microbes. Regardless of the mechanism, the ability of these variants to control a response to *Puccinia* rusts (and other microbes) in a non-specific manner make them interesting from an agricultural standpoint. While their necrotic phenotypes are too severe to utilize them directly, their occurrence indicates it may be possible to identify, or create, novel genes or gene combinations at complex loci that might exert pathotype non-specific control to pathogens.

There is preliminary evidence that pathotype non-specific resistance may be possible at *Rp1* in the absence of a severe necrotic phenotype (Hu and Hulbert, unpublished). Several *Rp1* haplotypes have been created which carry two or more *Rp1* genes linked in coupling, which can then be genetically manipulated as though they were a single gene. Some of these 'compound genes', such as *Rp1-JD4* (carrying *Rp1-J* + *Rp1-D*) have a slight necrotic or chlorotic spotting phenotype associated with them at the adult plant stage in certain genetic backgrounds. Randomly chosen F_3 families carrying *Rp1-JD4* were significantly more resistant at the adult plant stage than families not carrying the genes when challenged with the rust pathotype HI1, which is virulent on both genes. It is difficult to determine whether this resistance is pathotype non-specific, especially since HI1 is the only isolate available that is virulent on both genes. Additional experiments should determine if this form of resistance is really non-specific, if *Rp1-JD4* is unusual in this respect, or if other compound genes show a similar effect.

Conclusion

The majority of the naturally occurring variants that have been identified at the *Rp1* complex of maize arose by recombination events. The extent of the

reassortment via recombination (crossing-over and gene conversion) that is possible between *Rp1* genes is presumably enhanced by the tandemly duplicated structure of the complex. This allows frequent mispairing between different genes in an array leading to unequal crossing-over events. The consequence of these events in generating novel specificities at a high frequency in certain crosses may be the manifestation of an evolutionary mechanism designed to allow the plant to adapt rapidly to constantly changing pathogen populations. Sometimes these recombination events result in genes, such as the *Rp1*-lesion mimics, which, because of their extreme phenotypic effects, have an adverse effect on the plant.

While mispairing and recombination (crossing-over and conversion) are apparently events which can create diversity at *Rp1*, it should also be noted that these events are often considered to be the main driving force which homogenizes repeated sequences in the genome, causing both repetitive elements (Smith, 1976; Brutlag, 1980; Li and Graur, 1991) and members of small gene families (Hickey *et al.*, 1991) to be more alike within a species than between related species that once shared the repeats. Thus, the same forces that cause concerted evolution appear to play a role in the generation of diversity. It is probable that this diversity is maintained among resistance gene families by the selection for novel resistance types as a consequence of variation in pathogen populations.

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Mutation Analysis for the Dissection of Resistance

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It was demonstrated in 1905 that the trait of 'resistance' in wheat to *Puccinia striiformis* can be formally described by Mendel's laws indicating simple monogenic control (Biffen, 1905). Since then, we have learned that monogenic control of resistance is a common feature in essentially any intensively studied plant-pathogen interaction. Flor's studies with flax on resistance to *Melampsora lini* (rust) led to the gene-for-gene hypothesis, which turned out to be broadly applicable to cases in which resistance is controlled by monogenic, dominantly or semidominantly acting resistance genes (Flor, 1955, 1971). A central term in his model is interdependence in the sense that the simultaneous presence of a dominant pathogen function (avirulence gene) and a corresponding dominant host function (resistance gene) are necessary to initiate a successful defence response. Biochemically, Flor's gene-for-gene resistance has been frequently interpreted as a specific recognition event of a pathogen determinant by the plant triggering subsequently a defence response in the host. Therefore, a gene-for-gene interaction is likely to represent a signal-response coupling event. It is, however, difficult to apply Flor's model and its biochemical interpretations to the increasing number of documented cases of monogenic, recessively inherited resistance in plants (Adams and Somerville, 1996; Holub *et al.*, 1996; Schoenfeld *et al.*, 1996).

Biochemical studies of defence reactions in response to pathogen attack have identified a plethora of physiological changes and putative host compounds that may contribute to the resistant phenotype. They include a rapid induction of localized tissue collapse at the site of infection (the hypersensitive response, HR), release of preformed or *de novo* synthesized antimicrobial substances, a toughening of the plant cell wall, the oxidative burst, and the

accumulation of defence-related proteins (Lamb *et al.*, 1989; Bowles, 1990; Bradley *et al.*, 1992; Brisson *et al.*, 1994; Levine *et al.*, 1994; Mittler and Lam, 1996). Recently, several resistance genes following Flor's gene-for-gene mode of inheritance have been molecularly isolated (Martin *et al.*, 1993; Bent *et al.*, 1994; Jones *et al.*, 1994; Mindrinos *et al.*, 1994; Whitham *et al.*, 1994; Grant *et al.*, 1995; Lawrence *et al.*, 1995; Song *et al.*, 1995). The surprising finding is that the deduced proteins share remarkably similar structural domains although they trigger resistance reactions to pathogens such as viruses, fungi, and bacteria (Dangl, 1995; Staskawicz *et al.*, 1995). The isolated genes code for proteins that either encode a simple serine/threonine kinase or contain a leucine-rich region (LRR), with or without an attached nucleotide binding site (NBS), indicative of ligand-binding and protein-protein interaction. A structural combination of LRR and the kinase domain has been reported in the deduced protein from the rice *Xa21* resistance gene (Song *et al.*, 1995).

The structural similarity of resistance genes in gene-for-gene defence makes the existence of a common (or few) underlying resistance mechanisms very likely; but how can resistance genes trigger a seemingly diverse set of biochemical defence responses in a coordinated manner? If a resistance gene represents one component of a stimulus-response coupling event, one might ask how many additional host genes participate in the signal transduction before the biochemical reply is activated. Further questions arise as to whether the signalling is linear and/or branched, whether feedback mechanisms exist, if resistance genes represent the first step in these pathway(s), and how specificity is achieved.

Mutational analysis of resistance reactions may provide one way to answer some of the questions. Different laboratories have begun to identify genetically the components required to establish a resistant phenotype. The aim of this review is to survey the different mutational screens that have been used in different plant/pathogen interactions, to evaluate what has been learned with respect to signalling of resistance responses, and whether components might have escaped the present selection schemes of mutational gene identification.

Genes Required for the Function of Resistance Genes in Gene-for-Gene Interactions

Pioneering work has been performed by Torp and Jørgensen who analysed the genetics of resistance in the interaction between barley and *Erysiphe graminis* f. sp. *hordei* (Torp and Jørgensen, 1986; Jørgensen, 1988). The authors mutagenized a barley line carrying the resistance gene *Mla-12* using chemical mutagenesis with ethylmethanesulphonate (EMS) and sodium azide (NaN_3) and screened for susceptible mutants in the M_2 generation. A total of 25

susceptible individuals were isolated with an average frequency of approximately 0.3×10^{-3} . The phenotypes were in most cases not fully susceptible but showed a range of different infection types between the resistant phenotype of the mutagenized line and a susceptible wild-type cultivar. Test crosses of the susceptible mutants both with the resistant (*Mla-12*) and a susceptible (*m1a-12*) line showed that 22 mutants carried a defect within the resistance gene because all of the F_2 progeny in the test cross with the *m1a-12* line exhibited susceptible phenotypes. In contrast, only three mutants segregated both resistant and susceptible F_2 individuals in the latter test cross indicating that the *Mla-12* gene and a second defective locus, required for its function, could be separated by recombination. Thus, the mutational study revealed an unequal distribution of isolated defective alleles in the resistance gene and in genes required for its function (approximately 8 : 1). Further analysis showed that the three mutants required for *Mla-12* function represent two complementation groups. The corresponding loci have been designated *Rar1* and *Rar2* (required for *Mla-12*-specified resistance; former designation *Nar-1* and *Nar-2*; Freialdenhoven *et al.*, 1994). *Rar1* has been mapped on barley chromosome 2 and *Rar2* on chromosome 5 tightly linked to *Mla-12* (Freialdenhoven *et al.*, 1994; unpublished results).

A similar study has been conducted in tomato to identify mutations in genes required for resistance to the phytopathogenic bacterium *Pseudomonas syringae* pv. *tomato* (Salmeron *et al.*, 1994). Resistance to this pathogen depends upon the presence of the *Pto* locus in the host which recognizes pathogen strains expressing the avirulence gene *avrPto*. The *Pto* gene was the first race-specific resistance gene to be isolated and has been shown to encode a serine/threonine kinase (Martin *et al.*, 1993). The mutational analysis of *Pto*-controlled resistance also provides important clues to the serendipitous finding that tomato cultivars carrying *Pto* rapidly develop small HR-like flecks upon exposure to the organophosphorous insecticide, fenthion (Laterrot, 1985). The gene controlling fenthion sensitivity has been termed *Fen* and maps to the same locus as *Pto* (Carland and Staskawicz, 1993; Martin *et al.*, 1994).

Fenthion sensitivity has been instrumental in the mutational study of *Pto*-resistant tomato lines because it revealed that from a total of eleven susceptible mutants, six retained sensitivity to the insecticide and five became insensitive. As with barley mutants susceptible to powdery mildew infection, the degree of susceptibility in the tomato mutants varied 200-fold as determined by *in planta* bacterial growth of the *avrPto*-containing *Pseudomonas* strain. Genetic test crosses of the fenthion insensitive class of susceptible mutants with a susceptible (*pto*) line revealed increased resistance in the F_1 in each case compared with the mutant parents. In contrast, the other susceptible mutant class, characterized by retained fenthion sensitivity, showed equal or more severe symptoms in the F_1 of the test cross (Salmeron *et al.*, 1994). This suggested that the susceptible mutant class with abolished fenthion sensitivity carries defects in *Pto*, whereas the other class carries defects in a different gene termed *Prf*

(*Pseudomonas* resistance and fenthion sensitivity). Contrary to the study in barley, mutations in *Pto* and *Prf* were recovered with similar frequencies. The analysis was complicated by the fact that *Prf* is tightly linked to *Pto* and the loci could not be separated by recombination. In fact, if the susceptible mutants could not have been differentiated from each other by their fenthion sensitivity/insensitivity, it would have been difficult to demonstrate that the mutations reside within separate genes. The analysis sheds light on a general problem: a gene required for the function of a resistance locus is difficult to detect solely by test crosses of mutants if the loci are tightly linked.

Apart from the identification of *Prf*, the analysis described provided strong evidence that *Pto* and *Fen* are encoded by separate genes since all *Pto* mutants retained fenthion sensitivity. This was confirmed recently through the cloning of the *Fen* gene which encodes a protein sharing 80 per cent identity with *Pto* (Martin *et al.*, 1994). Both genes are members of a multigene family and were shown to be physically located on one isolated yeast artificial chromosome.

Molecular marker-based mutant screens

The degree of susceptibility in host mutants of plant–bacteria interactions can be easily quantified by monitoring bacterial titres at various time points after inoculation. Susceptibility of host mutants in plant–fungus interactions is more difficult to quantify. The pathogen usually passes through a series of developmental stages before it enters the final reproductive phase. Aberrant resistance responses in host mutants may be manifested only by an increased growth of vegetative fungal mycelium. These altered phenotypes might be detected with the naked eye in plants attacked by ectoparasitic but not endoparasitic fungi.

A sensitive mutational screen that does not rely on a macroscopic inspection of phenotypes has been developed in the tomato–*Cladosporium fulvum* interaction (Hammond-Kosack *et al.*, 1994). In this case eight mutants exhibiting reduced fungal resistance were recovered after chemical mutagenesis of a tomato line carrying the *Cf-9* resistance gene in a screen that involved a fungal isolate expressing the corresponding *avr9* avirulence gene. An *avr9* containing *C. fulvum* strain was utilized that constitutively expresses high levels of β -glucuronidase (GUS) activity both *in vitro* and *in planta* (Oliver *et al.*, 1993). Instead of screening for sporulating fungi on M_2 individuals, 4-methylumbelliferyl β -D-glucuronide (MUG) assays were performed directly on infected plant material. The MUG levels represent a measure of fungal biomass in cotyledon segments of inoculated seedlings. The sensitivity of the MUG test enabled a screening of tissue segments from pools of 25 M_2 individuals of a M_2 family. The GUS-based test enabled the identification of two classes of mutants that gave rise either to fungal sporulation or to increased vegetative mycelial growth.

The latter class would have been difficult to detect with the naked eye. Six mutants mapped to the *Cf-9* locus whereas two reduced-resistance mutants mapped at two distinct loci (*Rcr-1* and *Rcr-2*; required for *Cladosporium* resistance) unlinked to the resistance gene. As with cases described in tomato and barley, mutations in *Cf-9* showed highly variable infection phenotypes ranging from increased vegetative mycelium to full sporulation of the pathogen. Unlike the *Rar* mutants in barley, both *Rcr* mutants were only associated with growth of vegetative fungal mycelium but not completion of the fungal life cycle by sporulation. Thus, it seems likely that the *rcr* mutant alleles would have escaped detection if the screen had been based upon a macroscopic inspection of mutant seedlings.

Following injection of a race-specific elicitor peptide preparation into *Cf-9* wild-type plants, a characteristic necrotic response is observed in cotyledon tissue. Interestingly, all of the fully susceptible mutants at *Cf-9* had lost the ability to trigger the necrosis response upon elicitor injection, whereas the partially susceptible mutants including both *Rcr* mutants revealed a reduced necrosis response. This finding confirmed that mutations in *Cf-9* and *Rcr* are both due to alterations in a *Cf-9*-dependent defence response and not simply to a general increase of the plant's susceptibility to *C. fulvum* infection. Recently, additional *Rcr* genes have been identified in a tomato line carrying the *Cf-2* resistance gene (Dixon *et al.*, 1996; M. Dixon and J. Jones, personal communication). Because the mutagenized line contains two functional copies of *Cf-2*, an enrichment for mutations in genes required for *Cf-2* function was achieved. Two recessive and allelic *Rcr* mutants, each supporting full fungal sporulation, have been isolated among 900 M₂ families.

Another instructive example of a directed mutational search for genetic components of race-specific resistance has been performed in *Arabidopsis thaliana*. Screens have been carried out after inoculation of mutagenized resistant accessions with the fungus *Peronospora parasitica* and the bacterium *Pseudomonas syringae* pv. *tomato*. The search for mutations in genes required for *RPP5*-specified resistance to *P. parasitica* was successful in two accessions, Landsberg *erecta* (Ler-0) and Wassilewskija (Ws-0), each carrying different *RPP* specificities (Parker *et al.*, 1996; J. Parker, personal communication). The selection was directed towards loss of *RPP5* function in Ler-0 and towards loss of *RPP14* function in Ws-0. Apart from mutations in *RPP5* and *RPP14*, recessive mutations in a single locus designated *eds1* (enhanced disease susceptibility) were revealed in both accessions. Two allelic *eds1* mutants were isolated in Landsberg in comparison with six defective alleles in *RPP5*. One confirmed *eds1* allele (plus three possible but not fully characterized *eds1* alleles) and a single *RPP14* mutant were recovered in the Ws-0 screen. The findings indicate that *eds1* is required for at least two *RPP* functions (see below) and reveal that mutations in *eds1* can be at least as frequently isolated as in the *RPP* loci.

Immersion inoculation of fast-neutron mutagenized seeds of the resistant accession Columbia (Col-0), recognizing race-specifically a *Pseudomonas* strain

carrying *avrB*, led to the discovery of one susceptible mutant carrying a defect in a locus termed *NDR1* (non-race-specific disease resistance, see below for details; Century *et al.*, 1995). Three additional *ndr1* alleles have been isolated in a separate mutagenesis experiment involving a *Pseudomonas* strain carrying both *avrRpt2* and *avrB* (R. Innes, personal communication). This screen also detected three susceptible mutants that are neither allelic to *NDR1* nor to each other.

Some mutagenesis experiments have, in contrast to the cases described above, failed to detect mutations in genes required for resistance gene function. Fast neutron or gamma-irradiation of a lettuce line carrying resistance genes *Dm1*, *Dm3*, *Dm5/8* and *Dm7* to *Bremia lactucae* enabled the isolation of 16 susceptible mutants. Without exception, all of the mutants were shown to carry defects in the *Dm* resistance loci. Among those were nine independent inactivations in *Dm3* (Okubara *et al.*, 1994). Although it could be argued that radiation-induced mutagenesis might have been inappropriate to recover weakly defective alleles in putative genes required for *Dm* function, extended experiments using chemical ethylmethanesulphonate (EMS) mutagenesis again revealed only mutations in the *Dm* loci (R. Michelmore, personal communication). Similarly, an extensive screen for susceptible mutants in *Ara-bidopsis* to *P. syringae* carrying *avrB* revealed 12 allelic mutations that reside, without exception, within the *RPS3* gene (Bisgrove *et al.*, 1994; R. Innes, personal communication). The mutagenesis included radiation- and chemically-induced mutations. Although the mutagenesis did not detect mutations in genes required for *RPS3* function, it provided convincing evidence that a single resistance gene can specify resistance responses to two distinct avirulence genes (*avrB* and *avrRpm1*). All of the *rps3* alleles simultaneously lost the ability to recognize *avrRpm1*, making it very likely that *RPS3* and *RPM1* are encoded by the same gene. This has been confirmed subsequently by the molecular isolation of the *RPM1* gene and sequencing of some of the mutant alleles (Grant *et al.*, 1995).

Interactions between host genes required for resistance

Epistatic relationships between a pair of genes or between mutations in a regulatory or biochemical pathway may provide information about the way the genes interact without a priori knowledge of their molecular identity (Ferguson *et al.*, 1987; Chory, 1990; Roman *et al.*, 1995). Frequently, a large number of resistance specificities have been characterized in a single host plant both to related and different taxonomic groups of pathogens. It is therefore particularly interesting to study the interaction of genes required for resistance gene function in combination with various resistance gene specificities. The results have provided the first clues to the existence of common and distinct

components of responses and phenotypes triggered by different resistance genes.

The *ndr1* mutant in *Arabidopsis* mentioned above was identified by its requirement for the function of the *RPS3* gene. When this mutant was tested with *Pseudomonas* strains carrying avirulence genes that are recognized by resistance genes *Rpm1*, *Rpt2* and *Pph3* in accession Columbia (Century *et al.*, 1995), it supported equivalent levels of bacterial growth *in planta* to that of a virulent *Pseudomonas* strain lacking any of the four avirulence genes tested. This strongly suggests that *NDR1* represents a common component of a pathway activated from resistance genes *RPS3/Rpm1*, *Rpt2* and *Pph3*. The *ndr1* allele may, however, only partially inactivate the *RPS4* resistance function (K. Century, personal communication). Furthermore, the *ndr1* mutant supports increased sporulation of several *P. parasitica* isolates incompatible in the wild-type as determined by the number of sporangiophores per cotyledon. The fungal isolates tested are recognized by resistance specificities *RPP2*, *RPP4* and *RPP7* in accession Columbia. Thus, *NDR1* represents a common component of resistance responses to bacteria and a fungal pathogen.

Requirement of the *eds1* gene in *Arabidopsis* for the function of *RPP* specificities other than *RPP5* and *RPP14* has been tested. The emerging picture is that *eds* alleles inactivate several (*RPP1*, *RPP10*, *RPP12*) but not all *RPP* specificities (see Holub, Chapter 1 this volume). Interestingly, the *RPP8* specificity on chromosome 5 does not appear to be compromised in the presence of a defective *eds1* allele. This observation may indicate that different resistance specificities to the same pathogen use different signalling routes. Alternatively, the observed differential inactivation may be due to residual activities of *eds1* alleles. Surprisingly, mutant lines of *Arabidopsis* carrying *eds1* support growth of isolates of *P. parasitica* derived from *Brassica oleracea* which are incompatible in the wild-type *Arabidopsis thaliana*. This observation may provide evidence that components of race-specific resistance are also involved in so-called non-host resistance.

A similarly complex picture is emerging from gene interaction studies between the barley *Rar1* mutant alleles and various powdery mildew resistance loci. *Rar1* is located on barley chromosome 2 whereas the resistance gene *Mla-12*, which had been used in the mutation study to identify the *Rar* genes, maps to the tip of chromosome 5. Because a large number (31) of resistance specificities has been described for the *Mla* locus (Kintzios *et al.*, 1995), it was interesting to test interactions between the two available defective *rar1* alleles (*rar1-1* and *rar1-2*) and different *Mla* resistance specificities. Interestingly, several but not all of the 11 tested *Mla* specificities become inactivated in the presence of defective *rar1* and *rar2* alleles (Jørgensen, 1996). No differences were detected between the patterns of retained/abolished specificities at *Mla* in the presence of either *rar1* or *rar2*. In addition, similar inactivation patterns were observed for both *rar1* mutant alleles, *rar1-1* and *rar1-2*. Furthermore, the function of powdery mildew resistance loci unlinked

to *Mla* are also compromised (*Mlh*, *Mlk*, *Mlra*, *MIRu2*) by *rar1* and *rar2*. The data strongly indicate a common function for *Rar1* and *Rar2* in resistance which is activated differentially by different powdery mildew resistance loci.

We wanted to know whether *rar1* defective alleles partially inactivate resistance specificities in cases where we failed to detect macroscopically an interaction with a resistance locus (*Mlg*, *mlo*). A prerequisite for these studies has been a marker-assisted selection of the appropriate genotypes in F₂ generations from crosses of the *rar1* mutants with cultivars carrying diverse powdery mildew resistance specificities. Quantitative microscopic evaluations of single fungal interaction sites on barley leaves at early time points after inoculation detected no interaction in *rar1/mlo* and *rar1/Mlg* individuals, as measured by the frequency of fungal host cell penetration and the appearance of a characteristic single-cell HR (Peterhaensel *et al.*, unpublished). The latter is intriguing since *Mla-12*-specified resistance reactions are also associated with a single-cell HR of penetrated host cells, and mutations in either *Mla-12*, *Rar1* or *Rar2* abolish this cell death response in the first host cell penetrated (Freialdenhoven *et al.*, 1994). Thus, *Rar1* is required to activate the cell death response in the context of *Mla-12* but not *Mlg*.

The gene interaction studies described above all share one shortcoming. It is not known whether the available mutant alleles in genes required for the function of resistance genes represent null alleles or retain residual activity. If the latter is the case, the observed differential inactivation pattern of resistance gene functions could be explained on the assumption that some resistance genes require for their function wild-type activities of NDR, *eds1* Rcr or Rar proteins, whereas others tolerate diminished activity or act through a different domain in the same protein. These uncertainties will only be resolved once the corresponding genes have been isolated.

Genetic Control of Race Non-Specific Resistance: a Case Study

Recessive alleles of the *mlo* resistance gene in barley confer a race non-specific resistance response to almost all powdery mildew isolates (Jørgensen, 1994; Lyngkjær *et al.*, 1995). Resistance alleles at *Mlo* can be induced by mutagenesis of any susceptible cultivar so far tested. At the cytological level the resistance response is associated with a quantitative arrest of fungal germlings in a subcellularly restricted cell wall apposition prior to haustorium development (Jørgensen and Mortensen, 1977). No cell death response can be detected in attacked host cells. Interestingly, *mlo* plants grown under sterile conditions exhibit a constitutive expression of the defence response as indicated by a high frequency of spontaneous cell wall apposition formation in the epidermal target tissue (Wolter *et al.*, 1993). Thus, it has been proposed that the *Mlo*

wild-type allele may function as a negative regulator of the race non-specific resistance. Alternatively, the wild-type allele could represent an as yet unidentified compatibility factor for this obligately biotrophic fungus (Johal *et al.*, 1995).

A mutational approach has been used to identify genes required for *mlo* function (Freialdenhoven *et al.*, 1996). The mutagenesis uncovered two loci, *Ror1* and *Ror2* (required for *mlo*-specified resistance). Five recessive mutant alleles (and two further not fully characterized alleles) were recovered for the *Ror1* gene and a single defective allele for *Ror2*. Plants carrying each of the mutant alleles exhibit infection phenotypes intermediate between *mlo*-resistant and *Mlo*-susceptible lines. Mutations in the *Ror* genes abolish the function of different *mlo* resistance alleles tested and confer susceptibility to various *mlo*-avirulent powdery mildew isolates. At the cytological level, a 20–30-fold increase in fungal penetration frequency into the first host cell attacked was observed. The mutagenesis experiments support a model in which the *Mlo* wild-type allele acts as a negative regulator of a race non-specific resistance and in which the *Ror* genes have a positive regulatory function. Thus, a single resistance response may be subject to both negative and positive genetic control.

An interesting question was whether or not plants carrying mutant *ror* alleles retained the capability to express race-specific resistance. Experiments have been conducted with genotypes carrying either *Mla-8* or *Mlg* resistance alleles together with defective *ror* alleles (C. Peterhaensel and A. Freialdenhoven, unpublished). All of these genotypes showed a fully resistant phenotype at the macroscopic level after inoculation with isolates carrying either the corresponding *avrMla-8* or *avrMlg* avirulence function. Marker-assisted selection of the respective genotypes enabled a microscopic evaluation at early time points after inoculation and indicated that the plants retained the capability to activate the characteristic timing of *Mla-8*- and *Mlg*-associated defence responses. Because the mutant *rar1* alleles, which abolish the function of several race-specific powdery mildew genes, do not compromise the function of *mlo* alleles, it was concluded that race specific and race non-specific resistance to the same fungal pathogen operate through genetically distinct pathways. The *Ror1* gene has recently been mapped close to the centromere on barley chromosome 5, confirming its distinct map position both from *Rar1* and *Rar2* (C. Peterhaensel, unpublished).

A Fatal Connection: Deregulated Tissue Necrosis and Enhanced Resistance

One common attribute of the plant defence response is the appearance of a spatially restricted tissue necrosis during pathogen attack, which is assumed to

confine pathogenic growth within the collapsed tissue. It has been suggested that a class of mutants, termed lesion mimics (*Les*) or necrotic mutants (*nec*), affect the control of the defence response (Neuffer and Calvert, 1975; Walbot *et al.*, 1983; Pryor, 1987). There has been speculation that at least some of the dominantly acting *Les* mutants in maize represent alleles of resistance genes that activate the defence response in the absence of a pathogen-derived elicitor. This has been confirmed recently in an extensive study of the *Rp1*-complex of maize conferring resistance to *Puccinia sorghi* in which four mutants or recombinant *Rp1* alleles were found to exhibit a lesion mimic phenotype (Hulbert and Bennetzen, 1991; Hu *et al.*, 1996). Similarly, the mutation-induced *mlo* powdery mildew resistance alleles in barley exhibit spontaneous formation of cell wall appositions in leaf epidermal cells, resembling those formed in response to a bona fide fungal attack (Wolter *et al.*, 1993). At later time points during seedling development, the plants develop leaf necrotic flecks, even when grown under aseptic conditions.

Another intriguing example is the *sl* mutant in rice that has been termed Sekiguchi lesion (Marchetti *et al.*, 1983). The lesions are first visible as 1- to 2-mm-diameter spots that enlarge rapidly, and coalesce later until the whole plant is affected. Sekiguchi lesions can be induced by avirulent but not virulent isolates of *Bipolaris oryzae* or *Pyricularia oryzae* or by exposure to chemical agents such as organophosphate insecticides. Histological analysis of *Bipolaris oryzae*-inoculated resistant wild-type and *sl* mutants revealed no evidence of pathogen proliferation from the primary inoculation sites. These findings suggest that the *sl* mutation identified a gene that limits the spatial extent of the HR.

Recessively inherited lesion mimic mutants have been systematically analysed in *Arabidopsis* (Greenberg and Ausubel, 1993; Dietrich *et al.*, 1994; Greenberg *et al.*, 1994). The affected genes have been designated *acd* (accelerated cell death; *acd1* and *acd2*) or *lsd* (lesions simulating disease resistance response; *lsd1* to *lsd5*). Each of the mutants exhibits, in the absence of pathogens, HR characteristics such as plant cell wall modifications and the accumulation of defence-related gene transcripts. Leaves of the *acd2* mutant have been shown to accumulate high levels of salicylic acid and of the *Arabidopsis* phytoalexin, camalexin (Tsuji *et al.*, 1992). Application of low levels of salicylic acid or its structural analogues induced lesion formation in the *lsd1* mutant. Importantly, *acd* and *lsd* mutants exhibit elevated resistance to a bacterial (*P. syringae*) and fungal (*P. parasitica*) pathogen. The *lsd1* mutant is exceptional in that it confers heightened pathogen resistance at a prelesion state, in contrast to the other defective loci which exhibit elevated pathogen resistance only in the lesion-positive state. In this respect, *lsd1* resembles the *mlo* mutants in barley. Another striking feature of *lsd1* is the indeterminate spread of lesions in contrast with the other mutants where lesion growth is determinate. In this respect, *lsd1* is similar to the rice *sl* mutant.

Genetic Dissection of Acquired Resistance

Systematic genetic screens have also been initiated to identify components controlling inducible resistance mechanism(s) in plants. Systemic acquired resistance (SAR) develops in distal, uninfected parts of a plant after a primary challenge with an avirulent pathogen as first shown in the tobacco/tobacco mosaic virus interaction (Ross, 1961a, 1961b). SAR has been shown to be widespread in plants in response to a primary challenge with any pathogen that causes necrosis. The conferred resistance is against a typically broad spectrum of viral, bacterial and fungal pathogens (Kuc, 1982; Uknes *et al.*, 1992; Kessman *et al.*, 1994). Chemicals such as salicylic acid (SA), 2,6-dichloroisonicotinic acid (INA), and benzothiadiazole (BTH) can mimic pathogen-induced SAR after exogenous application to plants (White, 1979; Metraux *et al.*, 1991). The accumulation of endogenous SA seems to be necessary for the expression of the SAR phenotype as well as the accumulation of a wide range of pathogenesis-related proteins (Yalpani *et al.*, 1991; Enyedi *et al.*, 1992; Alexander *et al.*, 1993; Delaney *et al.*, 1994).

A mutant screen has been established in *Arabidopsis* after exogenous SA application (Bowling *et al.*, 1994; Cao *et al.*, 1994). For the selection of SAR-defective mutants, transgenic *Arabidopsis* plants were generated containing the bacterial GUS gene under the control of the β -1,3-glucanase promoter. The β -1,3-glucanase gene represents one out of several pathogenesis-related genes whose expression is upregulated after SA, INA or biologically induced SAR. EMS-mutagenized M₂ individuals were screened for SA- or INA-non-responsive mutants based on GUS activities found in tissue samples from 15-day-old seedlings grown in the presence of SA or INA. A total of 77 'non-expresser' mutant lines have been identified out of approximately 14,000 M₂ plants tested. One recessive mutant, *npr1* (non-expresser of PR-genes), has been characterized in detail (Cao *et al.*, 1994). The mutant exhibits a 10-fold lower expression level of the chimeric GUS gene as well as a lowered endogenous β -1,3-glucanase gene expression compared with wild-type plants. In addition, a 20-fold reduction in *PR-1* gene expression was observed. Importantly, SA- and INA-mediated resistance to the virulent bacterium *P. syringae* was reduced 1000-fold as measured by bacterial growth *in planta*. However, inoculation with a *P. syringae* strain carrying the avirulence gene *avrRpt2* revealed a typical HR, as indicated by the rapid appearance of tissue collapse and accumulation of autofluorescent substances in the infected cell walls. Therefore, the *NPR1* gene is a necessary component of the SAR, but is seemingly not required for race-specific resistance responses. Interestingly, *PR* gene expression was absent in peripheral regions of lesions after inoculation with a virulent strain of the bacterium. The lesions in *npr1* plants were also found to be more diffuse and spatially extended in comparison with *NPR1* wild-type plants, suggesting that *PR* gene expression might not only function to restrict

pathogen growth in distant parts of an infected plant but also to restrict the proximal spread of pathogens in infection sites.

A variation of the screen described above has been applied to identify mutants that constitutively express the chimeric GUS reporter gene construct (Bowling *et al.*, 1994). One recessive mutant, *cpr1* (constitutive expresser of PR genes) has been isolated that shows not only elevated expression levels of the chimeric β -1,3-glucanase gene but also increased expression levels of the endogenous β -1,3-glucanase gene, the *PR-1*, and *PR-5* genes. The mutant was found to confer elevated resistance both to a virulent strain of the bacterial pathogen *P. syringae* and a virulent isolate of the fungus *P. parasitica*. Endogenous levels of free SA and the sugar conjugate, SA β -glucoside were 4, 5- and 21-fold higher in *cpr1* compared with wild-type plants. Expression of the *nahG* gene, encoding the bacterial salicylate hydroxylase (You *et al.*, 1991; Gaffney *et al.*, 1993), neutralizes the constitutive PR gene expression of *cpr1* plants. It was, therefore, concluded that the *CPR1* gene acts upstream of SA. The findings strongly suggest that the wild-type *CPR1* allele acts as a negative regulator of SAR in *Arabidopsis*. Because the *NPR1* gene is supposed to act downstream from SA, it would be interesting to test this proposed gene order by constructing a *npr1/cpr1* double mutant. In this genotype one would expect an epistatic action of *npr1*.

In a similar study, *Arabidopsis* mutants failing to respond to SA-induced resistance were sought as measured by subsequent assays for resistance to *P. parasitica* (Delaney *et al.*, 1995). A recessive mutation, *nim1* (non-inducible immunity), insensitive to both chemical and biological inducers of SAR, has been described in detail. As with the *npr1* mutant, *nim1* exhibits diminished expression of pathogenesis-related gene expression upon SA application or pathogen inoculation. In contrast to the *npr1* mutant, *nim1* plants supported growth of two isolates of *P. parasitica* incompatible on the wild-type. Thus, *NIM1* might have a common function in SAR and genetically determined resistance. Because *nim1* mutants retain the capability to accumulate wild-type levels of endogenous SA, it has been suggested that the wild-type *NIM1* gene acts downstream of SA accumulation but upstream of genetically determined resistance and SAR-mediated gene expression.

The role of salicylic acid and the functional overlap of SAR and genetically determined resistance has been explored further in *Arabidopsis* and tobacco by studying pathogen responses in transgenic lines that constitutively express the *nahG* gene (Delaney *et al.*, 1994). 'Hypersusceptibility' was detected both to virulent bacterial and fungal pathogens in the *nahG* transgenic lines compared with wild-type plants. For example, the bacterial titre of a virulent strain of *P. syringae* pv. *tomato* was 10 to 50 times greater in the *nahG* transgenes than in the non-transgenic *Arabidopsis* line. Importantly, race-specific resistance was also almost completely abolished. This has been tested by using a bacterial *P. syringae* pv. *tomato* strain expressing *avrRpt2* that is recognized by the corresponding resistance gene *Rpt2* in accession Columbia. The bacterial titre in the

nahG transgene carrying *Rpt2* was four to five times greater than in the non-transgenic line and almost identical to the titre measured after inoculation with *P. syringae* pv. *tomato* in the absence of *avrRpt2*. The resistance response could be restored after application of INA prior to pathogen inoculation. The findings imply that SA accumulation not only has a crucial role in SAR but is also important in race-specific resistance, on condition that the *nahG*-mediated SA depletion in the transgenes does not have profound secondary effects on the plant metabolism.

Conclusions

Although somewhat limited, the available data from mutational screens of 'gene-for-gene' mediated resistant plants in *Arabidopsis*, tomato and barley reveal obvious similarities. Only a few loci were uncovered in each case. This might indicate that the number of genetically identifiable components in the putative signalling pathways is low. The findings contrast with the number of loci detected in other plant signal-response coupling events. At least 14 loci are involved in the expression of the triple response phenotype in the presence of ethylene (Ecker, 1995). Mutant screens unravelled at least 10 loci involved in abscisic acid signalling (Giraudat, 1995). More than 20 genes regulate flowering time in response to the environmental stimuli of day length and temperature after germination (Coupland, 1995). Currently, one can only speculate whether this is due to the fact that ethylene, abscisic acid, and day length each participate in the control of multiple aspects of plant growth and development, whereas a gene-for-gene resistance response is rapid, affects usually few cells, and is highly specific.

In general, genetic dissection of phenotypes is limited in application to non-redundant components of pathways. There is a suggestion that saturation mutagenesis has been achieved in a few mutant screens because of the repeated isolation of defective alleles at the same locus. However, the screens have until now not included a systematic approach to the recovery of lethal mutants. More importantly, it seems likely that a refinement of the screening procedures, enabling the detection of subtle alterations to resistant phenotypes (e.g. the GUS-based assay to detect vegetative fungal mycelium in the tomato/*C. fulvum* interaction), will uncover additional loci. An alternative route to mutation analysis has been recently reported to reveal components of resistance reactions (Zhou *et al.*, 1995). The yeast two-hybrid system enabled the identification of a serine/threonine kinase, *Pti1*, that physically interacts with the tomato *Pto* protein specifying resistance to bacterial speck disease. The functional contribution of the *Pti1* gene to the resistance response was shown by *Pti1* transgenes in tobacco exhibiting an enhanced HR in an *avrPto*-dependent manner. Thus, in cases where a defence component is present in multiple copies in the genome, this approach will contribute to building a

complete picture of the signalling pathway when mutation analysis fails to do so. In conclusion, although the number of genetically identifiable components in the putative signalling pathways of gene-for-gene resistance appears to be low, the actual number could be large if redundancy is prevalent.

Another conclusion to be drawn from the gene interaction studies is that each of the identified loci appears to participate in resistance responses activated by different resistance genes. This may not be too surprising given the observed structural similarity of domains in deduced protein sequences from many race-specific resistance genes. If a single, conserved biochemical mechanism operates in race-specific resistance responses, it seems plausible that *NDR1*, *eds1* and *Rar1* represent signalling components rather than 'effector genes' at the end of a signal pathway, because mutant alleles of the three loci abolish several but not all race-specific resistance functions. Disappointingly, however, the present data do not provide clues with respect to gene order. *NDR1*, *eds1* and *Rar1* may either act up- or downstream from race-specific resistance genes. Only if the simplest biochemical model is applied, a ligand-binding activity of resistance genes, is it plausible to assume a downstream position for *NDR1*, *eds1* and *Rar1*.

Mutation analysis of SAR has clearly provided a link with genetically determined resistance as shown by the *nim1* mutant. In the case of the *eds1* mutant, an overlap between the control of race-specific and non-host resistance was revealed. Thus, mutation analysis challenges the traditional terminology used to characterize various forms of plant resistance responses. On the other hand, different resistance responses can be separated from one another (as shown by the *ror*, *rar* and *npr1* mutants) indicating the existence of separable signal transduction pathways. It is tempting to deduce models from these studies but we refrain from this temptation because, particularly with respect to gene order, reliable data are currently not available.

Have the current mutant screens overlooked a group of genes? At least in gene-for-gene resistance there has so far not been a systematic attempt to uncover negative regulatory genes. The role of this class of genes has generally been underestimated in signal-response coupling events (Bowler and Chua, 1994). Because gene-for-gene resistance is a signal-response coupling event, it seems very likely that negative regulatory components exist (see also the proposed negative regulatory function of *CPR1* and *Mlo*). The present mutant screens have had a bias towards defects in positive regulatory genes, but they might provide the basis for a next generation of screens: genotypes containing weakly defective alleles of genes required for resistance gene function in combination with functional copies of resistance genes could be mutagenized and screened for (partially) restored resistance. This might provide an enrichment for loss of function mutants that negatively control the speed or the spatial extent of a defence response. It will be interesting to see whether and to what extent such mutants provide new links; perhaps providing an explanation of lesion mimics.

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Cultivar Mixtures in Intensive Agriculture

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Modern intensive agriculture demands the highest cost-effective yields even if inputs have to be very high. Low-cost and highly effective pesticides, particularly fungicides, have meant that choice of cultivar has been constrained little by inherent disease susceptibility. Uniformity of quality and response to management practices have also demanded the use of monocultures. However, pressure on profit margins and increased concern about pesticide usage from an environmental point of view, have encouraged renewed emphasis on genetic disease resistance and its effective deployment.

Where disease resistant cultivars are used, the consequence of such monoculture over large areas is enhanced selection for pathogen genotypes able to overcome the resistance, resulting in the classical 'boom-bust' cycle of cultivar usage. Until early this century local bulk-selected landraces were used which comprised diverse disease resistances (van Leur *et al.*, 1989; Broers and Dehaan, 1994). Agriculture in general was more diverse and such mixed cropping has been shown to provide a buffer against environmental variables such as disease (e.g. Bonman *et al.*, 1986, in Wolfe and Finckh, 1996). Such observations have led to the use and development of mixtures in many agricultural systems. In this chapter I will address their use in intensive agriculture where, hitherto, they have attracted only minimal interest. I will attempt to summarize: (1) what mixtures are, (2) their attributes and use, (3) how mixtures work—particularly (4) which effects are 'agronomic' and which 'genetic', (5) whether mixtures will remain effective, and (6) how mixtures might be improved.

What Cultivar Mixtures Are

The modern plant breeding process is expensive, wastes genetic diversity which could be of value to a crop, and tends to result in few single resistance genes being selected (Johnson, 1961). This can be compensated for by diversification schemes encouraging different resistance genotypes to be grown in neighbouring fields. Multilines – near-isogenic lines back-crossed until the only identifiable characteristic in which they differ is pathogen resistance – were devised as a way of effectively trying to mimic the resistance diversity of landraces while retaining the uniformity of monocultures in other respects (Browning and Frey, 1969; Wolfe, 1985). However, multilines were even more expensive and complex to breed than single cultivars. Therefore, interest moved to mixtures of cultivars which vary for many characters including disease resistance, but have sufficient similarity to be grown together (Wolfe, 1985). Species mixtures are also exploited in intensive agriculture; for example, combinations of some or all of wheat, barley, oat and peas in Poland (Czembor and Gacek, 1996; Daellenbach *et al.*, 1996), and agroforestry is being implemented in specialist situations. However, cultivar mixtures are perhaps the most easily exploited to produce some gain as they need cause no major changes to the agricultural system, except to reduce pesticide inputs. They are quicker and cheaper to formulate and change than multilines (Wolfe, 1973; Wolfe and Barrett, 1977) and can be formulated to have multiple and diverse disease resistance genetic types which will provide more protection and be more difficult for a pathogen to overcome (Wolfe, 1973, 1978; Wolfe and Barrett, 1977). The heterogeneous host genetic backgrounds may slow down the development of a 'super race', and have greater buffering capacity against other environmental factors (Allard, 1960; Wolfe, 1978; Wolfe and Barrett, 1980).

The Attributes and Use of Cultivar Mixtures

In the presence of disease, mixtures of cultivars frequently yield more than the mean of the components grown as monocultures (Finckh and Mundt, 1992a; Czembor and Gacek, 1996; Gacek *et al.*, 1996a,c). In the 1980s, in the former German Democratic Republic (GDR), most of the spring barley was grown in mixtures (approximately 300,000 ha) as a practical means of reducing pesticide inputs while maintaining high yield and quality for beer production (Wolfe, 1992; Wolfe and McDermott, 1994). Good control of mildew, and coincidentally brown rust, was achieved using only three mildew resistance genes and field sizes of 50 to 100 hectares.

In Poland approximately 60,000 ha of spring barley mixtures were grown in 1995 (Czembor and Gacek, 1996) and while the yields of the mixtures were not much higher than the means of the components, they were much more

stable between environments (Gacek *et al.*, 1996a,c). Mixtures of barley cultivars have been used successfully for many years in Denmark and are increasing in popularity in Switzerland (Merz and Wolfe, 1996). In the USA around 100,000 ha of cultivar mixtures and near-isogenic lines of wheat are grown (Wolfe and Finckh, 1996).

Yield stability is thought to be one of the main attributes of mixtures (Allard, 1960; Wolfe and Barrett, 1980). This is most simply illustrated by comparing the variance of the mixture with its components; it is usually less (e.g. Allard, 1960; Dubin and Wolfe, 1994). Others have used more precise tests using geometric and regression analyses (Dubin and Wolfe, 1994; Daellenbach *et al.*, 1996; Gacek *et al.*, 1996b).

How Cultivar Mixtures Work

Chin and Wolfe (1984a) proposed three different mechanisms that delayed pathogen multiplication and spread in mixtures relative to monocultures. The first two mechanisms are physical. The dilution effect results from increasing the space between plants of the same genotype, relative to monocultures, which reduces the number of spores from the plant of origin reaching other like plants (alloinfection). Barley mildew (*Erysiphe graminis* f. sp. *hordei*) spread has been demonstrated to be density dependent (Burdon and Chilvers, 1976). The barrier effect results from interruption of spore movement by plants of a resistant or non-host genotype. Both these mechanisms can be manipulated by plant density and the number of different mixture components.

Resistance reactions induced by the avirulent spores may delay or prevent infection by the neighbouring virulent spores when avirulent pathogen spores are deposited in the same vicinity of the leaf. The effect was shown to be an important component of control exerted by cultivar mixtures for powdery mildew of barley (Chin and Wolfe, 1984a) and yellow rust (*Puccinia striiformis*) of wheat (*Triticum aestivum*) (Lannou *et al.*, 1995) accounting for about 20% of the total disease reduction achieved in the mixture. However, these responses were very variable, probably because the induced resistance affects only a few host cells around an attempted infection site.

All these effects are dependent upon the type of pathogen, particularly its mode of infection and dispersal. The gradient of dispersal, a factor critical in spatial models used to analyse mixture effects, is particularly important.

Which Effects of Cultivar Mixtures Are 'Agronomic' and Which Are 'Genetic'

A well designed mixture incorporating several different major genes for resistance to the target pathogen, such as mildew in barley, will consistently

out-perform the mean of its components grown in monoculture. However, the fungicide treated control may do likewise, indicating a strong beneficial 'agronomic' mixing effect. For example, in 1989 a barley mixture comprising Doublet, Tweed and Natasha caused a 15% reduction in mildew compared with its monoculture components and yielded 10% more. However, the fungicide-protected control also gave a 6% yield increase (Fig. 4.1) so only about 4% of the mixture advantage was attributable to disease control (Newton and Thomas, 1991).

Yield competition and compensation

Whilst control of disease is the main focus of most mixtures studies, to concentrate solely on this characteristic will result not only in a failure to recognize other important characteristics that should be developed, but also may result in misinterpretation of mixtures effects. Reports of such yield increases in mixtures can be explained by yield competition and compensation (Allard, 1960; Finckh and Mundt, 1992a,b).

Plants such as barley exhibit much plasticity in their responses to the environment, which may be a further benefit of mixtures. Thus the performance of a mixture will differ between each environment as components respond in different ways. This is another feature of mixtures which can be developed by selection of components for particular environmental responses. Two kinds of competition act in mixtures: intragenotypic and intergenotypic (Jolliffe *et al.*,

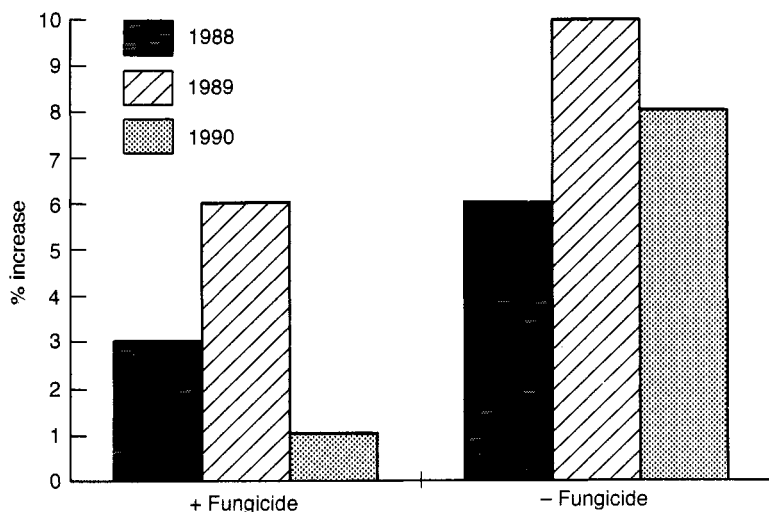


Fig. 4.1. The effect of fungicide treatment of a major gene mixture of spring barley cultivars on yield over three successive years.

1984). If the intragenotypic competition is greater than the intergenotypic competition, the mixture will yield more than the mean of the monoculture controls. However, the interaction of the components with each other is as important as the interaction with environment.

Morphology

Disease reduction may result not only from genetic interactions between host and pathogen, but also in physical interactions. Diversity in plant morphological types is often likely to result in better resource utilization both above and below ground. A denser, more stratified canopy structure may result in less air movement in the canopy, restricting spore transmission. This may also retain a higher humidity thus promoting infection. In the case of a splash-dispersed pathogen, many more niches in the canopy are likely to be filled thus reducing vertical splash dispersal as illustrated in Fig. 4.2. While the increase in genetic complexity of the mixture, as more components are used, can be accounted for by the classical genetic explanation, increase in morphological heterogeneity is likely to reinforce this effect in the case of a splash-dispersed pathogen such as *Rhynchosporium secalis*. Interestingly, the relationship between disease control and component number was consistent between years but yield response was not, again reinforcing the importance of other mixture interactions.

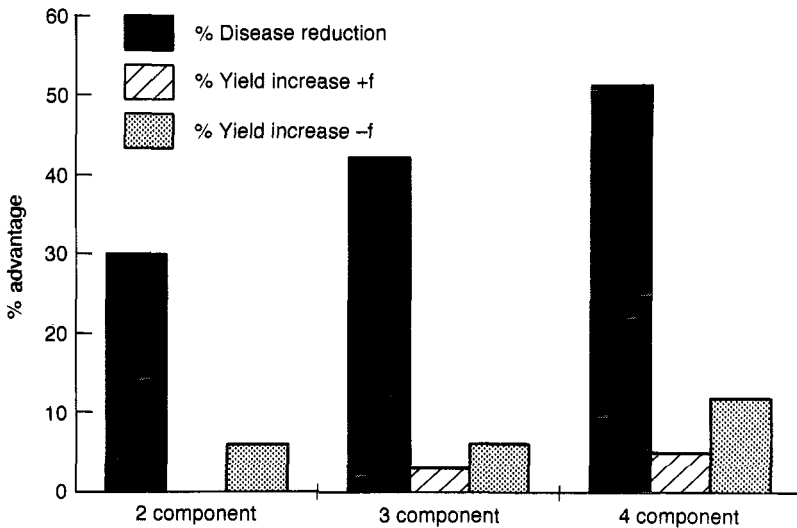


Fig. 4.2. The effect of increased component number of winter barley cultivars on *Rhynchosporium secalis* infection, and on yield in the presence and absence of fungicide in mixtures. -f = no fungicide, +f = fungicide.

Polygenic resistance

Because of the prevalent use of major genes to control the main pathogens of intensive agriculture, diversification has been based on these genes from both a theoretical and practical point of view. Polygenically-based partial resistance, has been largely ignored in developing cultivar mixtures, although it has been studied in the control of diseases which express less cultivar specificity such as *Rhynchosporium secalis* and *Septoria (Stagonospora) nodorum* (Jeger *et al.*, 1981a,b). However, most work on cultivar mixtures has concentrated on the the rusts and mildews of cereal crops which exhibit marked cultivar specificity. Nevertheless, even in these diseases, polygenic resistance can be effective, reducing disease and increasing yield, though generally not as much as major genes. The lack of high levels of disease control in such mixtures also revealed a relationship between greater yield loss of the components of a mixture in monoculture and advantage gained in mixtures (Newton and Thomas, 1991). This is presumably a yield competition/compensation response, whereas gains resulting from disease control obtained with near-isogenic lines are in proportion to the effectiveness of the resistance (Kølster *et al.*, 1989). The instability of performance from year to year of mixtures using polygenic resistance reinforces this point (Newton and Thomas, 1993). A combination of polygenic or non-specific resistance together with specific resistance can work well (Wolfe *et al.*, 1981; Newton and Thomas, 1993).

Pathogen population

Consideration of agronomic versus genetic effects should not be limited to the host population as the value of particular specific resistances in cultivar mixtures depends primarily upon the frequency of matching virulences in the pathogen population. Mixtures providing greater advantage were constructed from cultivars for which pathogen genotypes able to overcome more than one component were relatively uncommon in the pathogen population in comparison with cultivars for which matching races were already common (Martinelli, 1990, in Wolfe and Finckh, 1996; Newton and Thomas, 1991). Thus knowledge of pathogen population structure is important.

Experimental factors

Several other factors affect the reproducibility of trials from year to year. For example, small plot sizes are poor at producing a mixture effect, as the epidemic needs time to develop and the edge effects in such plots are great (Gieffers and Hasselbach, 1988). Inoculum pressure is likely to be higher in small plots and in all mixtures trials this will vary both within and between seasons. By

manipulating inoculum pressure in a checkerboard pattern of either guard plots or inoculum-producing plots, it was possible to demonstrate that under low inoculum pressure, infection was reduced by 47% in mixtures whereas under high pressure there was no reduction (A.C. Newton, 1996, unpublished results). However, the 47% disease reduction produced no yield increase, whereas under high inoculum conditions an 8% yield increase in the mixtures was recorded. Presumably these effects are explained by poorly understood yield compensation and competition effects.

Another common complicating effect is nitrogen or nutrient status in general. Again, available nitrogen will vary from year to year and in a given year it can be demonstrated that 'low' nitrogen results in good mildew reduction on barley in mixtures and results in large yield increases, whereas under high nitrogen there is no overall disease reduction in the mixtures and only a modest yield increase (Newton *et al.*, 1996). However, very different disease reduction and yield responses were obtained from the same trial carried out in the following year.

Will Cultivar Mixtures Remain Effective?

In the former GDR, mixtures of barley were used comprising only three resistance genes. To produce a 'super race', the pathogen needed to recombine only three virulences, two of which, *Va12* and *Va13* were common across Europe, to produce a 'super race'. No virulence towards the third resistance gene, the durable *mlo* gene, has yet been detected in Europe. An increase in the recombinant genotype with *Va12* and *Va13* was found in the GDR in 1990, but the pathogen isolates with this gene combination originated from Poland and the former Czechoslovakia (Wolfe *et al.*, 1992). This pathotype presumably had no fitness advantage as it did not dominate, and simple races tended to predominate at the start of each season (Schaffner, 1993, in Wolfe and Finckh, 1996).

Polygenic or 'race non-specific' resistance is perceived to be 'durable' and does not result in selection for matching virulence in the pathogen population. If this is true, there should be no reduction in disease levels in mixtures of cultivars expressing only this type of resistance, but this is not so (Newton and Thomas, 1991), although whether the effect is attributable to matching virulence rather than some other preference of pathogen isolates for particular host genotypes as growth substrates, is unknown. However, this serves to underline the importance of considering all aspects of a plant's genotype as a suitable component of a mixture. Disease control can be achieved by the action of many genes in addition to those which trigger specific resistance mechanisms.

Whatever the mechanism, pathogen adaptation to a genotype expressing resistance of any type is undesirable and may result in erosion of the

effectiveness of such resistance when used either alone or in mixtures. There is evidence for such adaptation not only towards 'conventional' major resistance genes (virulence), but also to hitherto durable resistance genes such as *mlo* in spring barley (Lyngkjær *et al.*, 1995), and to polygenic or 'partial' resistance to mildew in spring barley (Newton, 1989, 1992; Newton and McGurk, 1991). Even so, such resistance erosion is likely to be slowed by its use in mixtures compared with monocultures. Mundt (1994) concluded that, despite theoretical considerations, complex races do not tend to dominate the pathogen population in mixtures and the rates of change that do occur would allow appropriate changes in the mixture composition to be effected.

Considering all the criteria listed above, good mixtures composed of the best cultivars available frequently give disappointing yields which are little if at all greater than the mean of their components, leaving only stability across environments as their main attribute. This is often due to there being little effective diversity of resistance available for use in mixtures, the pathogen population being able to overcome readily all components. This is probably why the accumulative data from large scale mixture trials in Poland demonstrate only very modest yield increases, which some consider fail to offset the disadvantages of mixtures (Gacek *et al.*, 1995b). The problem is probably accentuated by the fact that some resistance genes appear to result in selection for non-corresponding virulence genes (Wolfe *et al.*, 1983; Huang *et al.*, 1995b).

The evolution of 'super races' with a genotype able to overcome all resistance components in a mixture has long been considered a danger. There is evidence of selection for increased virulence complexity in mixtures even during a growing season. For example, the virulence complexity of individual field isolates increased by an average of over 10% in just 4 weeks in such a field trial of spring barley mixtures sampled recently at SCRI (A.C. Newton, 1996, unpublished results). Chin and Wolfe (1984b) and many other workers (Wolfe, 1984; Dileone and Mundt, 1994; Huang *et al.*, 1994, 1995a) also found that barley mixtures selected for more complex powdery mildew races than did monocultures. However, because of the epidemiological effect of mixtures, complex genotypes did not necessarily increase in absolute numbers relative to their increase in monocultures. Chin and Wolfe (1984b) also found that different barley cultivars containing the same race-specific resistance caused disruptive selection into subraces of the pathogen which were differentially adapted to the genetic background of the cultivars. Dileone and Mundt (1994) found that selection for an increase in the number of complex pathotypes in mixtures was inversely related to the number of other pathotypes occurring in the mixture. Thus, the overall diversity within the pathogen population as a whole rather than in single isolates may be the most important factor. In normal monocultures there is apparently little selection for complex or 'super races' of a pathogen able to attack several cultivars, and simple races able to attack single cultivars predominate (Wolfe and McDermott, 1994; Caffier *et al.*, 1996, in Wolfe and Finckh, 1996).

How Cultivar Mixtures Might be Improved

Modelling and experiments

Our ability to predict the performance of mixtures and thus to design more effective mixtures is still highly subjective and lacks precision. Clearly our understanding of all the processes involved is incomplete. One way round trying to understand all the processes involved is to build either simulatory or analytical models and subsequently test their validity in the field (see Mundt, 1989 for a review).

Experiments (Mundt and Browning, 1985; Mundt and Leonard, 1986) and computer simulations (Mundt *et al.*, 1986; Mundt and Brophy, 1988; Goleniewski and Newton, 1994) indicate that the number and the size of the host genotype unit area are important parameters, but intimate mixing may not be essential for optimal restriction of disease. If there is a need to control several pathogens, the best planting strategy will probably be that which best controls the pathogen with the shallowest dispersal gradient. Therefore, strip planting could permit sowing and harvesting of the components separately, which may have practical advantages. Despite more disease in alternating strips of wheat than in random mixtures, disease was less than in monocultures and yield was more than in random mixtures (Brophy and Mundt, 1991). Wide strips also tend to reduce selection for complex races (Huang *et al.*, 1994, 1995a).

A feature of most models, as with real experimental data, is that mixtures are most effective in restricting disease during establishment on uninfected tissue early in the epidemic. Later in the epidemic the disease level in the mixture catches up with the mean disease of the components grown as monocultures as carrying capacity for the disease is approached. However, this is often late in the epidemic and a yield benefit is still achieved (Sitch and Whittington, 1983).

Resistance combination

While disease reduction and, therefore, selection of the best resistance combination, may seem the most important criteria for designing mixtures, basic agronomic characteristics must take higher priority. Mixture components must have very similar or complementary quality characteristics, planting and harvesting times. Resistance component choice will then be very restricted but should aim for maximum heterogeneity towards the target diseases. Polygenic or non-specific resistance together with specific resistance works well, and mixtures varying in both are best (Wolfe *et al.*, 1981). Even then the composition should be changed in a planned way from time to time. This may be dictated by new cultivars coming on to the market, which will make useful

mixture components. Data from pathogen surveys demonstrating the frequency of virulence gene associations will aid these decisions.

The main advantage of mixtures are to maintain a high level of yield (above that of the mean of the components) over a wide range of locations and seasons, i.e. stable production. Maximizing restriction of disease requires a mixture of the best current resistant components grown in such a way as to maximize interaction among them.

Combining ability

Cultivars used in mixtures should have good 'ecological combining ability', being both a 'good' competitor and a 'good' neighbour. Such cultivar pairs yield more when grown together than in monoculture. These interactions can be highly cultivar specific (specific mixing ability) but some show positive characters of general mixing ability. This distinction has been explored using combining ability analysis (e.g. Schutz and Brim, 1971; Knott and Mundt, 1990), although interaction effects may also differ with the number of components in the mixture.

Induced resistance

As the third major component of the mixtures effect, induced resistance is important in performance (Chin *et al.*, 1984). The degree of induced resistance varies with genotype (Martinelli *et al.*, 1993). There is evidence for differences in response to applied resistance elicitors in the field and controlled environments between cultivars of barley (Reglinski *et al.*, 1993; A.C. Newton, 1996, unpublished results). For example, a greater increase in papilla size in response to attempted infection by mildew was evident in the spring barley cultivar 'Proctor' following elicitor treatment than with 'Golden Promise' (A.C. Newton, 1996, unpublished data). There appears to be potential for more exploitation of induced resistance in mixtures and as a breeding objective in its own right. To enhance expression of induced resistance in mixtures, each specific resistance gene should be in a different component cultivar. This should insure the maximum number of avirulent genotypes and therefore the maximum induced resistance.

Quality

Mixtures can be used to improve quality where certain characters which could be of importance do not exhibit continuous variation in single cultivars, e.g. amylose : amylopectin ratios in starch, or pro-anthocyanin levels. By

incorporating different proportions of appropriate genotypes into a mixture, it would be possible to vary the expression of these characters and possibly achieve an optimum combination with other parameters.

For particular markets, such as the grain distilling market, wheat cultivar mixtures may have their greatest advantage over monocultures as they can be grown with low inputs of nitrogen and fungicides, thereby increasing gross profit margins. Strong, or possibly enhanced expression of one particular character may be required for other markets. Examples of this are soft wheats for grain distilling, high diastase barleys, also for grain distilling, and oat cultivars with high soluble beta-glucan, for improved fibre content in human nutrition. Even within pure cultivars, there will be variation between grains for such characters, owing to factors such as position on the ear. Within a mixture, the degree of heterogeneity will be increased, but this may not be important if a satisfactory mean level of expression is obtained.

Disadvantages

The main disadvantages of mixtures are the necessity to mix seed before sowing and the resistance of end-users to their purchase. End-users argue that they need to have pure cultivars in order to satisfy the appropriate qualities for their use. However, blind tests have demonstrated that, for malting quality, mixtures have proved highly acceptable (E. Gacek, Poland, 1995, personal communication).

Breeders do not in general work towards the selection of mixtures, partly because they must have access to a large number of cultivars in current production and in practice a cultivar's commercial importance is often short. The problem of new cultivars out-yielding mixtures presents a practical marketing problem. In Oregon, selection for mixture response is carried out, but this is unusual and likely to be effective only for the environment in which it is conducted.

The cost of the seed-mixing process is likely to be considerably less than the cost of fungicides, so if the yield benefits are equivalent, mixtures will be worthwhile. The highest sustainable yields could be obtained from mixtures plus some fungicide use, although this would not necessarily give the highest gross margins. Farm-saved seed cannot be used successfully as the mixture becomes unbalanced in composition. Therefore, sowing mixtures is suited to high technology agriculture where use of particular genes, chosen to manipulate the pathogen population, can be controlled closely.

Fungicides

Reduced-dose foliar fungicide applications can supplement mixing (De Vallavieille-Pope *et al.*, 1988), as can applying seed treatments to a single

component of a mixture (Wolfe, 1981; Wolfe *et al.*, 1987). Such techniques enhance disease control and may, in the case of seed treatment, increase diversity in the host population forcing disruptive selection. However, in practice such operations partially defeat the purpose of growing mixtures.

Field deployment

The cultivation method that maximizes disease restriction is maximum intimacy of the components. This may be disadvantageous where the components need to be harvested separately and later separation is impractical or too expensive. To achieve the maximum effect, the mixture should have maximum heterogeneity in both overall composition and spatial distribution. In practice, the best compromise of planting arrangements should facilitate separate harvesting of components, e.g. strips of different components. Just as a farmer normally has to compromise between yield and quality, with mixtures a further compromise among diversity in disease resistance, yield and quality characteristics must be considered. Past observations are not predictive of a future environment, so the safest strategy for the farmer is almost always to choose a high-yielding mixture instead of a monoculture.

The future

In many parts of the developing world, intraspecific mixtures are the norm, for example in upland rice (Bonman *et al.*, 1986) and in bean (*Phaseolus vulgaris*) (Trutmann *et al.*, 1993) cultivation. However, for mixtures use to increase in intensive agriculture there will probably need to be the stimulus of legislation restricting pesticide use or some other positive incentives. Alternatively, grain buyers will need to start accepting grain on the basis of its observed quality at delivery rather than on any concern over whether the grain came from a monoculture or a mixture.

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Crop Resistance to Parasitic Plants

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Striga ('witchweed') and *Orobanch*e ('broomrape') species are parasitic flowering plants. They infect the roots of many crops of economic importance in the Mediterranean regions, eastern Europe, the former USSR (*Orobanch*e) and sub-Saharan Africa (*Striga*). Yield losses can be up to 100% and are routinely 50% (Parker and Riches, 1993). Control of parasitic plants is difficult because the parasite life cycle is tightly linked to that of the host. All stages of parasite development are linked to chemical signals from the host. After about 10 days imbibition, usually when the rains start, parasite seeds need a stimulus from host roots to initiate germination, thus ensuring that only those seeds near to the roots germinate and, hence, are near to sites of infection. The other parasite seeds remain dormant. Parasite radicles penetrate host roots and parasite tubercles ('haustoria') develop on the host root surface. This organ facilitates transfer of nutrients and water from the host to the parasite. During the early stages in the parasite's life cycle whilst it is underground, it is completely dependent on the host, with maximum damage occurring to crop growth and subsequent yield. Once parasite stems emerge above ground, photosynthesis occurs in *Striga* species, but the majority of assimilates are still derived from the host plant (Parker and Riches, 1993). *Orobanch*e species are also parasitic and are achlorophyllous. Flowering and seed production is completed within 6 to 8 weeks after the emergence of parasite stems. Each parasite stem can produce 20 to 90,000 seeds depending upon the species. Parasite seeds can remain viable in the soil for up to 20 years, so effective strategies to control the parasite have to be immediately effective and of long-term duration.

Successful control of *Orobanch*e has been obtained with herbicides applied pre-emergence and with soil fumigants, but these options are only

economically feasible for high-value vegetable crops (Jacobsohn, 1994). No one method has yet been found to control *Striga* species. Resistant crops offer the potentially most effective and environmentally benign strategy for the control of parasitic plants. Resistance to parasitic plants is characterized by the absence or low number of emerged parasite stems (Ramaiah, 1987). Assessment of germ plasm has mostly focused on cowpea and sorghum for *Striga* species, and sunflower and faba bean for *Orobanch*e species. Fields infested with parasite seeds or soil mixed with parasite seeds in pots were usually used to screen germ plasm. Laboratory based systems have had a mixed reliability in predicting field resistance to parasitic plants (reviewed by Cubero *et al.*, 1994). However, those tests which assessed the ability of sorghum varieties to stimulate germination (Hess and Ejeta, 1992) and the responses of cowpeas inoculated with *S. gesnerioides* have been shown to reflect fully subsequent field performance (Cubero *et al.*, 1994; Moore *et al.*, 1995). The development of resistant varieties has been most successful in cowpea and sunflower, where it has been assisted by knowledge of the extent of variation of parasite virulence. Cowpea and sunflower form the principal focus of this review.

***Striga gesnerioides* – Cowpea**

Cowpea is an important staple food legume in West Africa, often providing the only source of protein in the diet (Aggarwal, 1991). Cowpea plants are also used as animal fodder. *Striga gesnerioides* is a serious constraint to cowpea production, resulting in yield losses of 30 to 50% (Aggarwal, 1991). Cowpea varieties with resistance to *S. gesnerioides* have been identified, characterized and are now being deployed in many countries of West Africa.

Sources of resistance

Cowpeas Suvita 2 from Mali and 5857 from Senegal were identified with resistance to *S. gesnerioides* in the early 1980s in field trials in West Africa by IITA (International Institute of Tropical Agriculture) (reviewed by Berner *et al.*, 1995). Additional resistance came from a landrace B301 from Botswana, which proved to be resistant to *S. gesnerioides* from 11 sites in West Africa in pot tests (Parker and Polniaszek, 1990). Cowpeas such as B301 and 5857 have poor seed and agronomic qualities. More recently, *in vitro* screening of 37 cowpea accessions revealed two resistant landraces from Niger (872) and Nigeria (APL 1) with good seed qualities (Fig. 5.1). These two cowpeas were subsequently shown to be resistant to *S. gesnerioides* in field trials in Mali (Moore *et al.*, 1995). It is noteworthy that all these resistant cowpeas did not support any successful parasite development.

Genetics and breeding for resistance

Three single dominant resistance genes to *S. gesnerioides* (*Rsg1*, *Rsg2* and *Rsg3*) have been characterized in varieties B301, IT82D 849 and Suvita 2, respectively (Atokple *et al.*, 1995; Singh and Emechebe, 1996). In addition, variety B301 carries duplicate dominant genes for resistance to another parasitic plant, *Alectra vogelii*. These genes are distinct from the *Rsg1* gene (Singh and Emechebe, 1996).

Since 1987, breeding programmes in West Africa have transferred resistance from the landrace germ plasm, e.g. B301, into elite and locally adapted cowpea varieties. Variety IT84S 22464 was used as the susceptible parent as it is high yielding and resistant to insects. In the late 1980s, there were several reports from West Africa of the 'breakdown' of B301 resistance. However, samples of *S. gesnerioides* from these locations were shown to be non-virulent

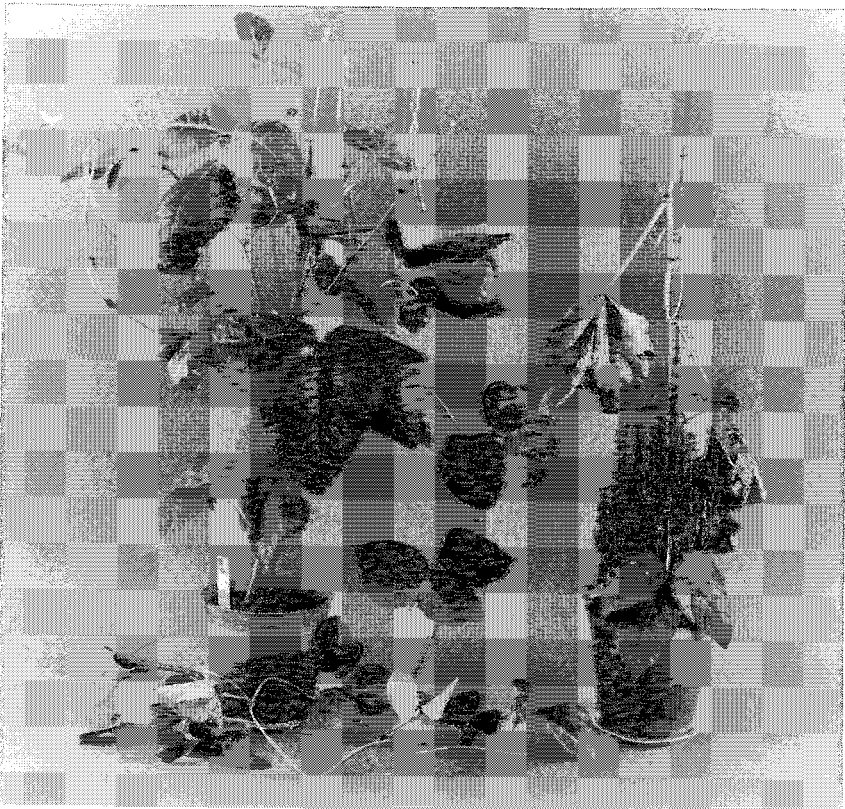


Fig. 5.1. Resistance to *Striga gesnerioides* in cowpea; susceptible cowpea variety Blackeye on the right-hand side and resistant variety 872 on the left-hand side.

on B301 plants from the type collection of the genotype (Lane and Bailey, 1992). It seems most probable that outcrossed B301 seed had been used in those trials. This confirmation of the validity of the effectiveness of the *Rsg1* gene was instrumental in the continued use of this gene in breeding programmes. In 1995, varieties based on the *Rsg1* gene were released in Nigeria (B.B. Singh, Accra, 1995, personal communication). Breeding lines (F₆) from these crosses have also been distributed across West Africa for use as parents in the transfer of resistance to locally adapted varieties.

Resistance mechanisms

Two distinct mechanisms of resistance to *S. gesnerioides* have been characterized in cowpea. In neither case was resistance associated with a lack of parasite germination or penetration of host roots by *S. gesnerioides* seedlings. In the first mechanism, *S. gesnerioides* seedlings penetrate cowpea roots but die within 3 to 4 days with an associated necrosis of host tissue around sites of parasite penetration (Lane *et al.*, 1993). This mechanism occurs in cowpea varieties 5857 and 872, and related legume species, including French bean, which are resistant to *S. gesnerioides* (Lane *et al.*, 1993, 1994b; Moore *et al.*, 1995). Preliminary studies revealed the presence of the phytoalexins: phaseollin, phaseollidin and phaseollinisoflavan, in the hypersensitive tissues of French bean roots infected by *S. gesnerioides* (Lane *et al.*, 1996a).

In the second mechanism of resistance, the development of *S. gesnerioides* on cowpea variety B301 is severely restricted, with parasite tubercles remaining at less than 1 mm in diameter, with limited stem development (Lane *et al.*, 1993). Xylem connections between host and parasite are thought to be essential for the flow of nutrients thus enabling successful parasite development. Ultrastructural studies showed that there was xylem–xylem contact between *Striga* and host cells on both B301 and susceptible cowpea roots within 4 to 5 days of placing parasite seedlings on host roots. However, the numbers of xylem strands and sieve tube elements in tubercles on B301 roots were far fewer than on the susceptible variety, Blackeye (Reiss *et al.*, 1995). Fluorescent tracers were added to host phloem cells to reveal the connections between host and parasite. These tracers remained in the central xylem strands of tubercles on B301 roots, whilst movement throughout central and peripheral xylem strands was observed in those tubercles on Blackeye. It was concluded that the reduced number of vascular connections between host and parasite on B301 roots may account for part of the observed reduction in parasite growth.

It was proposed that tubercle growth on B301 roots may be limited by an inadequate supply of endogenous plant growth regulators (PGR) from host roots to parasite tubercles. However, it appears that PGRs are not involved in the expression of resistance because the addition of exogenous auxins, cytokinins or gibberellins to B301 plants failed to stimulate tubercle growth. The

only detectable change was that limited parasite stem elongation was initiated by the addition of gibberellins (Reiss *et al.*, 1995).

Parasite variability and gene deployment

Variation in *S. gesnerioides* akin to that described in fungal–host interactions was first observed in field trials in Africa and was subsequently characterized using a differential series of cowpea varieties. Cowpea variety 5857 was resistant to *S. gesnerioides* in Burkina Faso but was susceptible in Mali and Niger (Aggarwal, 1991). Pot experiments with three resistant cowpea varieties and *S. gesnerioides* from 11 sites in West Africa identified three variants of *S. gesnerioides* (Parker and Polniaszek, 1990).

Knowledge that *S. gesnerioides* was widely distributed across West Africa (Cardwell and Lane, 1995) stimulated the need for a greater understanding of the distribution of virulence variants across the region. In the early 1990s, resistant cowpea varieties were being developed, so it became essential to know the geographic range of the variants in order to ensure the effective deployment of resistance. *Striga gesnerioides* was, therefore, collected from the main cowpea growing areas of seven West African countries (Cardwell and Lane, 1995). Forty-eight *S. gesnerioides* samples were inoculated on to a differential series of four cowpea varieties (Blackeye, 5857, IT81D 994 and B301) grown using an *in vitro* inoculation system (Lane *et al.*, 1991, 1996b). Five races were identified (Table 5.1) and their distribution across West Africa was mapped (Fig. 5.2). Parasite samples that developed only on cowpea variety Blackeye (race 1) were mostly from Burkina Faso but also from Mali, Nigeria and Togo. All other samples from Mali were pathogenic on varieties 5857 and Blackeye (race 2). *Striga gesnerioides* from Niger, northern and eastern Nigeria was pathogenic on varieties Blackeye, 5857 and IT81D 994 (race 3). Race 4 of the parasite from southern Benin was pathogenic on varieties Blackeye and B301. Race 5 was

Table 5.1. Races of *Striga gesnerioides* on cowpea.

Differential cowpea varieties	Races of <i>S. gesnerioides</i>				
	1	2	3	4	5
Blackeye	S	S	S	S	S
5857	R	S	S	R	R
IT81D 994	R	R	S	R	S
B301	R	R	R	S	R

S = Susceptible; parasite tubercles equal to or greater than 2.5 mm in diameter.

R = Resistant; parasite tubercles less than 2.5 mm in diameter and/or necrotic response present in cowpea roots, associated with death of *S. gesnerioides*.

pathogenic on varieties IT81D 994 and Blackeye and was identified among parasite samples from Cameroon, Nigeria, Benin and Burkina Faso.

Race 4 was the first variant with virulence on variety B301. Field trials also confirmed the susceptibility of B301 in southern Benin (Berner *et al.*, 1995). Varieties 5857 and IT81D 994 were resistant to race 4 (Lane *et al.*, 1994a). Resistance genes from these varieties have been pyramided into B301-derived progeny being developed for use in southern Benin (Singh and Emechebe, 1996).

***Orobanche cumana* – Sunflower**

Sunflower is a major oleaginous crop in eastern Europe and the former USSR and has been grown there for over 200 years owing to its role as a substitute for animal fat during Lent (reviewed by Sackston, 1992). More recently, sunflower has been grown for confectionary use in other parts of Europe. *Orobanche cumana* parasitizes sunflower causing serious yield losses and is distributed across the Mediterranean, eastern Europe and the former USSR. Variation for parasite virulence has been described in several countries, but it is far from clear to what extent the races in one country are identical to those in another country.

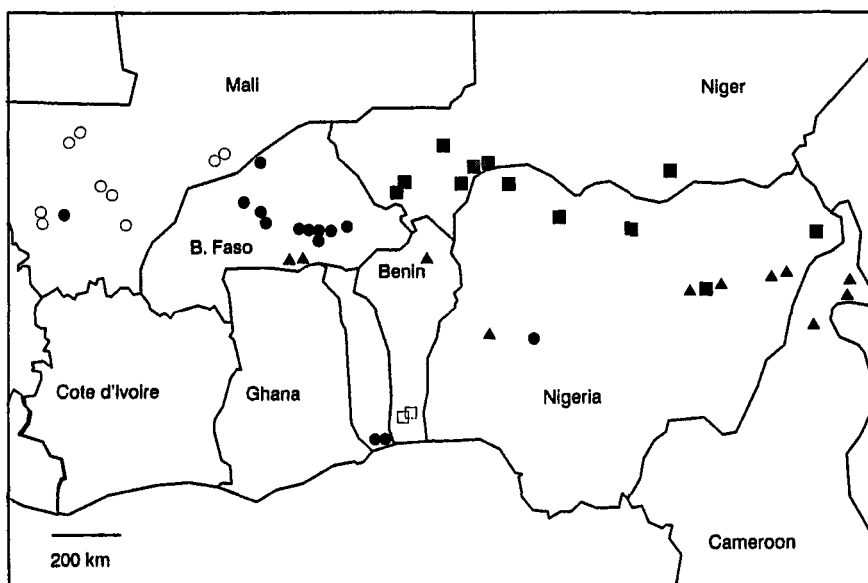


Fig. 5.2. Distribution of *Striga gesnerioides* virulence in West Africa. Race 1 of *S. gesnerioides* (closed circles); race 2 (open circles); race 3 (closed squares); race 4 (open squares) and race 5 (triangles).

Resistance breeding and parasite variability

Former USSR

The majority of the research on *O. cumana* and sunflower over the last 90 years has been conducted in the former USSR. Around 1900, sunflower became increasingly afflicted by *O. cumana*. As a result, local sunflower germ plasm with partial resistance to *O. cumana* was collected from farmer's fields in the Saratov region of middle Russia. Selections were initially based on field trials, and since 1921, also on pot trials (reported by Pustovoit, 1973). At this stage there was no knowledge of parasite virulence. Five resistant varieties, Kruglik A41, Saratovskii 169, Fuxinka 3, Zelenka 10 and Tchernyanka 35, were released over the period 1912 to 1928 (Pustovoit, 1973). In 1925, susceptibility of these varieties to *O. cumana* was reported from the Ukraine and Moldavia. Field trials in Krasnodar in southern Russia using *O. cumana* from these regions showed that there was variation in parasite virulence, so two races (A and B) were designated (Pustovoit, 1973). By the mid 1930s, race B had spread across all the sunflower growing areas of the former USSR and threatened to stop sunflower production (Pustovoit, 1973). Intensive efforts were made to locate resistance to the new race. For example, over 250 kg of *O. cumana* seeds were collected in the Krasnodar region in 1928 and sown in plots used for germ plasm assessment (Pustovoit, 1973). Resistance was found once again among landrace sunflowers collected from farmer's fields in the Ukraine. In 1934, varieties Zhdanov 8281 and 6432 with resistance to race B were released and were grown on over 1 million ha (Pustovoit, 1973). Varieties Peredovik and VNIIMK 1646 with resistance to both races and with a far higher oil content than the Zhdanov varieties were released in 1937. Between 1937 and 1960, resistant sunflowers were grown on over 5 million ha and *O. cumana* ceased to be a problem to sunflower production (Pustovoit, 1973).

In the 1960s, a new variant, C, with virulence on all resistant varieties was identified in Moldavia and the Ukraine. Race C is now prevalent across the sunflower growing regions of the south of former USSR (Antonova, 1994). Start and Odesskii 63, with resistance to race C, were identified in the 1970s by screening over 200 varieties in field trials in Moldavia (Buchuchanu and Karadzhora, 1984). Race D was first identified in the Krasnodar region in the 1990s. There is no resistance currently available to race D in the former USSR (Antonova, 1994).

Romania and Bulgaria

VNIIMK varieties from the former USSR with resistance to races A and B were resistant to *O. cumana* across most of Romania in the mid 1960s, except in the south-eastern region which borders Moldavia (Vranceanu *et al.*, 1986).

An analysis of virulence led to five *O. cumana* races being identified using a differential series of Romanian and former USSR sunflower varieties (Table 5.2) (Vranceanu *et al.*, 1986). Races D and E were characterized for the first time. A survey of six parasite samples from the coastal Black Sea and north-eastern regions revealed that races D and E were predominant (Vranceanu *et al.*, 1986). The variety Record was the first source of resistance to race C identified in Romania. Resistance to race D was found in variety S 1358, whilst the first source of resistance to race E was demonstrated in the inbred line P 1380 (Vranceanu *et al.*, 1986).

In Bulgaria, *O. cumana* was first observed in 1935 in the north-east of the country where it borders Romania and became a serious problem on sunflower by the 1940s. By 1950, over 85% of sunflower fields were infested with *O. cumana* (Entcheva and Shindrova, 1994). Races A and B were characterized in the 1950s using a differential series of sunflower varieties from the former USSR. Varieties Zhdanov 8281 and 6432 were introduced into Bulgaria in 1945 and 1952 respectively, because they were resistant to races A and B. These two varieties quickly became the most widely grown sunflowers in Bulgaria (Entcheva and Shindrova, 1994). Variety Peredovik was introduced in 1963 and was grown extensively because of its high oil content and resistance to *O. cumana*. Race C was identified in 1966 in the north-east of Bulgaria when Peredovik became susceptible. However, Peredovik continued to be grown over most of Bulgaria until 1985 because race C remained in the north-east and the southern Black Sea regions. In a recent study of *O. cumana* virulence, a differential series of sunflower varieties were inoculated with *O. cumana* samples from 12 sites across Bulgaria. Two more races, D and E, were identified for the first time in Bulgaria and were clustered along the north-eastern border with Romania (Shindrova, 1994). Races A and B are found in the north of Bulgaria, whilst race C was still confined to the Black Sea coastal region. Several new varieties, including Albena, Dobrich and Super Start, with resistance to races A, B and C were released in 1993. Variety Vega with

Table 5.2. Races of *Orobanche cumana* on sunflower in Romania.

Differential sunflower varieties	Races of <i>O. cumana</i>				
	A	B	C	D	E
AD 66	S	S	S	S	S
Kruglik A41	R	S	S	S	S
Zhanov 8281	R	R	S	S	S
Record	R	R	R	S	S
S 1358	R	R	R	R	S
P 1380	R	R	R	R	R

S = Susceptible; numerous parasite stems emerged in field trials.
R = Resistant; few parasite stems emerged in field trials.

resistance to all five races is now being grown in the north-eastern region of Bulgaria (Entcheva and Shindrova, 1994).

Turkey and Israel

Yield losses in sunflower of up to 50% were reported in Turkey over the period 1956 to 1962. The former USSR varieties Zhdanov 8281 and VNIIMK 8931 with resistance to races A and B were used successfully to restore yields until 1980, when new races became evident (Bulbul *et al.*, 1991). A differential series of seven Romanian and former USSR varieties were used to test *O. cumana* samples from across the northern Thrace region of Turkey which borders Romania. It was concluded that race E was present in that region but that the Romanian inbred, P 1380, and a Turkish variety 0043B were effective sources of resistance (Bulbul *et al.*, 1991).

Orobancha cumana was first found in Israel in the mid-1980s and is now considered to be the most rapidly spreading of all *Orobancha* species (Jacobsohn, 1994). Varieties with resistance to *O. cumana*, such as Sunbred 254, are used commercially. Breeding programmes in Israel focus on obtaining resistant confectionary sunflower varieties, which are usually much more susceptible to *O. cumana* than oleaginous varieties (Jacobsohn, 1994). Nothing has been reported about the races distribution in Israel.

Spain

Serious losses in confectionary sunflowers were first recorded in central and southern Spain in 1958 and *O. cumana* is presently regarded as the most important parasite of sunflower (Cubero, 1994). In 1979, Kruglik A41, Zhdanov 8281 and Peredovik were susceptible to *O. cumana*, suggesting that races D or E were present, but no differentials for these two races were used (Gonzales-Torres *et al.*, 1982). In another study, *O. cumana* was non-virulent on Zhdanov 8281 and P 1380, suggesting that an additional race, F, was also present in Spain (Melero-Vara *et al.*, 1989). A differential series of six Romanian sunflower varieties was recently used to assess the pathogenicity of *O. cumana* from 28 locations in southern Spain and most samples exhibited identical virulences to the putative race F (Saavedra del Rio *et al.*, 1994). Genetic variability within *O. cumana* was also revealed using isozyme markers (Castejon-Munoz *et al.*, 1991).

Breeding has focused on developing confectionary sunflower varieties using S 1358 as a resistant parent. Three new lines, R 2, RHA 273 and HA 99 have been developed (Saavedra del Rio *et al.*, 1994). Several USDA sunflower lines were also identified with resistance to *O. cumana* (Ruso *et al.*, 1994).

Genetics of resistance

In early Russian research, resistance to races A and B was usually found to be controlled by single dominant genes (described by Sackston, 1992). Five dominant resistance genes (*Or1–Or5*) were identified by analysing the progeny of 82 interspecific crosses among 110 varieties (Vranceanu *et al.*, 1986). *Or5* confers resistance to all five races, *Or4* to races A to D, *Or3* to races A to C, *Or2* races A and B, and *Or1* to race A only. Three Spanish sunflower lines carried single dominant resistance genes but the *Or* genes exhibited epistasis (Saavedra del Rio *et al.*, 1994). Resistance of the Israeli variety, Sunbred 254, is also controlled by a single dominant gene (Ish-Shalom-Gordon *et al.*, 1993).

Resistance mechanisms

A study of the infection process of *O. cumana* on resistant sunflower variety, Erdirne, from Turkey revealed that resistance was expressed after penetration of host roots (Dorr *et al.*, 1994). Germination of *O. cumana* and penetration of host roots was comparable on both resistant and susceptible sunflower varieties. Most *O. cumana* seedlings which penetrated variety Erdirne died with a necrotic reaction of host cells around sites of penetration. Ultrastructural studies revealed a densely stained layer of host cells formed around invading *O. cumana* cells. Increased lignification of host xylem elements was observed adjacent to *O. cumana* tissues. Thickened cell walls around *O. cumana* cells were detected in variety Sunbred 254 with an associated increase in total phenolic composition in these cells (Ish-Shalom-Gordon *et al.*, 1990). Research in the former USSR also revealed that lignin-like layers were present in resistant sunflower xylem cells in contact with *O. cumana* (Antonova, 1994).

The physiological compatibility of *O. cumana* on sunflower can be altered by changes in growing conditions, notably temperature. The resistance of sunflower variety Sunbred 254 to *O. cumana* observed in summer in Israel was not evident when it was grown in the cooler winter conditions (Ish-Shalom-Gordon *et al.*, 1994). A similar phenomenon has been described in plant–fungal interactions (Vanderplank, 1982).

***Orobanche crenata* – Faba Bean**

Faba bean is the only other crop infected by *Orobanche crenata* for which there has been any major effort to develop resistant varieties. Breeding in faba bean against *O. crenata* has mostly focused on an Egyptian variety, Giza 402 (reviewed by Cubero, 1994). Generally, resistance to *O. crenata* was polygenic and strongly additive, attributes which have reduced the emphasis on breeding as a solution to *O. crenata* on faba bean.

***Striga asiatica* and *Striga hermonthica* – Cereals**

Striga asiatica and *S. hermonthica* parasitize a wide range of cereal crops in sub-Saharan Africa, including maize, pearl millet and sorghum. Resistance to *Striga* of the type in which no parasite stems emerge has not yet been identified in any cereal. In sorghum, SAR varieties which stimulate parasite germination only weakly have been developed and variety SRN 39 has been released in Sudan (ICRISAT, 1991). Recently, SRN 39 and several SAR varieties were shown to have additional resistance mechanisms which were expressed after initial infection (Lane *et al.*, unpublished data 1996a).

Variation in the virulence of both *S. asiatica* and *S. hermonthica* has been suggested on the basis of different amounts of parasite emergence on resistant sorghums in field trials across Africa (Parker and Riches, 1993). Studies in which *S. hermonthica* from East and West Africa was inoculated on to the roots of a resistant sorghum variety IS 7777 (Olivier *et al.*, 1991) revealed differences in the virulence of these two parasite samples (Julian *et al.*, 1995). Molecular taxonomy studies confirmed the genetic differentiation of populations from East and West Africa (Bhrathalakshmi *et al.*, 1990). Thus, any new resistance genes will have to be deployed carefully on a regional basis. Data on the differential virulence of *Striga* species on cereals across Africa are needed urgently.

Conclusions and Future Directions

Similarities exist between the resistance of sunflower and cowpea to parasitic plants, but there are several major agricultural and historical differences. Research on sunflower and *O. cumana* has been conducted for over 90 years in the former USSR, whereas breeding for resistance in cowpea only commenced in the 1980s. The emergence of new *O. cumana* races and their subsequent movement across Europe has stimulated the development of resistant sunflower varieties. Similarly, three variants of *S. gesnerioides* were first characterized in 1990. This knowledge provided the impetus for locating resistance to the variant from Niger and Nigeria as all the original resistant cowpeas were susceptible to this race. Sunflower is grown in monoculture over vast areas in eastern Europe and the former USSR, and this undoubtedly favours the selection and spread of parasite races. Cowpea is usually grown in crop rotations and intercropped with cereals, dispersed across West Africa, thus reducing the possibilities for rapid multiplication of a new race (Cardwell and Lane, 1995). Resistance genes have yet to be deployed in cowpea widely across West Africa, but it seems unlikely that there will ever be the massive deployment of single resistance genes that typified control of *O. cumana* in the 1930s to 1960s in the former USSR.

In Africa, cowpea breeding is conducted mostly by IITA in collaboration with European laboratories. In sunflower, several countries have bred resistant varieties. Unlike the defined race structure and known distribution of *S. gesnerioides*, no such data exist for *O. cumana* (Lane *et al.*, 1996b). It is, therefore, not known whether *O. cumana* races in different countries are identical or dissimilar. Recent molecular studies of *O. cumana* from Israel and Spain showed that they were identical with regard to RAPD markers, perhaps indicating homogeneity of populations that have spread in recent times from eastern Europe and the former USSR (Katzir *et al.*, 1996).

New directions for resistance selection

The race structures of *S. gesnerioides* and *O. cumana* have become increasingly complex. Additional sources of resistance will be required to combat new races of both parasites. The proven sources of resistance are landrace material, e.g. cowpea variety 872 and landraces of sunflower utilized by Pustovoit (1973). Landraces can be exploited readily because they are often already adapted to local agronomic conditions (Moore *et al.*, 1995).

Wild relatives of crops are another good potential source of resistance genes. In sunflower, *H. tuberosus* (Jerusalem artichoke) was used in the former USSR to provide resistance to races A and B (reviewed by Sackston, 1992). Recently, 24 perennial *Helianthus* species were found to be resistant to *O. cumana*, whilst of the 16 annual *Helianthus* species tested only *H. anomalus* and *H. exilis* were resistant to the parasite (Ruso *et al.*, 1994). One wild relative of cowpea, *Vigna unguiculata* subsp. *mensensis*, is resistant to *S. gesnerioides* (Lane *et al.*, 1994c), but no use has yet been made of cowpea wild relatives in breeding programmes.

The chromosomal location of the resistance genes in cowpea or sunflower is unknown. In sorghum, a gene for low-stimulant resistance to *S. asiatica* was recently mapped using bulked segregant analysis and molecular markers (Weerasuriya, 1995). Marker-assisted selection, in conjunction with bulked segregant analysis, could be used to enhance the transfer of resistance from wild relatives into crop varieties, once molecular markers for the resistance have been identified.

The sympatric distribution of virulence variants influences successful resistance deployment. A suite of resistance genes will be required to encompass the parasite variation present in some countries. Resistance evaluation will have to be multilocal or use laboratory systems to rapidly assess new germ plasm in most countries. Use of *in vitro* systems could introduce new races, so such tests are probably most safely conducted in northern Europe where there are no quarantine problems. The *S. gesnerioides* distribution map (Fig. 5.2) will also be valuable for monitoring the movement of existing parasite races. The known resistant varieties are already being grown in test plots

throughout West Africa to assist in the detection of new races and the spread of existing races (Singh and Emechebe, 1996).

Evolution of new parasite virulences

In the former USSR, four *O. cumana* races have been identified over a period of 90 years, despite deployment of single resistance genes over 5 million ha. Two additional races have been characterized more recently from other countries. However, it should be noted that the rate of appearance of new parasite races is approximately once in every 20 years, a rate that is generally less than that found for pathogenic fungi. It is perhaps surprising that the races have not spread more rapidly, since some researchers moved *O. cumana* races into new countries or regions for field trials, with no mention of any phytosanitation procedures in order to prevent the escape of the exotic material (Pustovoi, 1973; Vranceanu *et al.*, 1986).

In West Africa, resistant cowpeas have only been widely grown in the last few years. Variety B301 was susceptible to *S. gesnerioides* in the first year that it was grown in southern Benin, thus refuting the possibility that the new race 4 arose following intense selection pressure imposed by growing the resistant variety (Lane *et al.*, 1994a). Samples of *S. gesnerioides* taken in 1983 and 1990 from a site in Mali had identical virulences, suggesting that new races do not arise very rapidly (Lane *et al.*, 1996b). All available evidence points to durability of resistance and for the slow rate of evolution of new parasite virulences in both *O. cumana* and *S. gesnerioides*. The stability of parasite populations probably reflects the soil habitat and single generation per year of these species, in contrast to pathogenic fungi where new races often appear rapidly.

Novel virulence variants may arise on alternative wild hosts. *Tephrosia* species are frequent wild hosts for *S. gesnerioides* in southern Benin, and may have been the origin of race 4 (Lane *et al.*, 1994a). In several countries, *Artemisia* species are wild hosts of *O. cumana* (Pujadas-Salva *et al.*, 1994). In Bulgaria, *O. cumana* parasitizes *A. maritima*, which commonly occurs in the Black Sea area where race C was first discovered (Entcheva and Shindrova, 1994). *Orobancha cernua* is morphologically very similar to *O. cumana* and is native to the Mediterranean region. Therefore, interspecies hybridization may also generate novel virulence variants. In the former USSR, *O. cernua* parasitizing *Artemisia* was recently shown to be virulent on sunflower variety Kruglik A41, so it was proposed that *O. cumana* evolved from *O. cernua* (E.S. Teryokhin, 1996, Bristol, personal communication). Molecular taxonomy of *Orobancha* and *Striga* species parasitizing wild and crop hosts would be highly instructive.

The resistance of cowpea and sunflower is under the control of single dominant genes but nothing is known about the genetics of parasite virulence. In the case of *S. gesnerioides*, the flowers are extremely small and short lived

making crosses between the races difficult (D.V. Child, personal communication). With the additional racial complexity of both parasites there is a need to resolve the origin and relatedness of parasite races and elucidate if a gene-for-gene relationship explains the observed race $\times$ variety interactions. A fuller understanding of parasite variation is required to direct the effective deployment of resistance against these complex biotrophic plants. Deployment of resistance has provided a successful strategy for control of parasitic plants, and with additional knowledge of parasite genetics, there is every prospect that this will continue to be the case.

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Population Genetics



The whole field of host–pathogen co-evolution is entering a new phase as a result of the recent cloning of the first resistance genes. Currently, agriculture makes extensive use of major genes for resistance in disease control strategies. Despite this, we know surprisingly little about the structure of these genes, their origin and mode of action, and the whole development of gene-for-gene interactions. This lack of information applies not only at the physiological and molecular level but also at the population and whole species level. Similarly, on the pathogen side we are still coming to terms with the complexities of the epidemiological and genetic behaviour of populations in field situations.

Understanding the dynamics of gene-for-gene systems from both a host and a pathogen perspective is essential for the development of effective long-term disease control strategies. However, restricting studies to agricultural systems (with their great ecological and genetic simplicity) limits an understanding of the basic structure of such interactions which originally evolved in the more complex ‘natural’ world. This section attempts to avoid these problems by considering in detail the extent of our understanding of the population genetics of host–pathogen systems, gleaned through the development of mathematical models based on population genetics theory tempered by epidemiological and life history considerations; studies of the population genetic structure of rust and mildew populations in agricultural situations; and finally from studies of natural host–pathogen interactions.

Many pathogens exhibit a range of reproductive strategies that have a profound influence on the genetic structure of their resultant populations. Populations trapped exclusively in an asexual mode of reproduction are likely to differ markedly from others in which periodic cycles of sexual reproduction

occur. In the former, the range of different pathotypes present in the population may be restricted, while highly unpredictable changes may occur in the frequency of virulence alleles not subject to direct selection. On the other hand, pathogens that indulge in periodic episodes of sexual reproduction may show a much wider diversity of pathotypes as a result of the generation of new virulence combinations through recombination. Even in these populations though, linkage disequilibrium between virulences under direct selection and those that are unnecessary, may rapidly develop as epidemics progress and the number of asexual generations following the sexual recombination phase increases. The nature of these and other interactions, and the complexities that they induce in pathogen populations is addressed in one form or another by Bayles *et al.*, Brown *et al.* and Kolmer who variously show the extreme fluctuations that occur in just a few years in the structure of populations of *Erysiphe graminis*, *Puccinia coronata*, *P. graminis* and *P. recondita*.

The mixed mating system shown by *Erysiphe graminis* is typical of many plant pathogens and, because of the complexities this introduces to an understanding and interpretation of population structure, Brändle and his colleagues have constructed a linkage map of the *E. graminis* genome in order to gain information on the chromosomal location of virulence loci under selection and other molecular markers. Using this they highlight the care needed in interpreting data obtained from markers for which linkage relationships are poorly known. Equally though, by using mating type alleles, they are able to address directly the question of estimating the proportion of sexual reproduction occurring in the fungal population.

Barley powdery mildew is also a very important disease across most of Europe and it is therefore not surprising that Hovmøller *et al.* viewed this system as an appropriate one on which to base a mathematical model aimed at investigating the mechanisms of host-induced selection and its influences on genetic changes in the pathogen population. Predicted changes in multilocus genotype frequencies were generally in accord with field observations, allowing the model to be used as a basis for assessing the consequences of different strategies of resistance gene deployment.

Contributions by Jeger and Leonard extend the modelling approach to a more general level. Jeger directs his interest to the possibility that life-history parameters may determine the long-term outcome of gene-for-gene systems and presents a model which integrates population genetics, life history and epidemiological approaches. Leonard, on the other hand, starts from the basis of a traditional population genetics model of the interaction between plants and pathogens by investigating a hard selection and a competition version of this model. From this he develops a comparison of resistance and virulence gene frequency dynamics in both a single pathogen population and one split into two subpopulations between which limited migration occurs.

Some of the guiding ideas and parameters used by Jeger and Leonard come from studies of the complexity and dynamics of natural host-pathogen associa-

tions. The last two chapters in this section provide examples of such systems. In a consideration of the interaction occurring between *Erysiphe fischeri* and *Senecio vulgaris*, Clarke shows just how heterogeneous both host and pathogen populations may be, and yet, because of the complex virulence phenotypes of most *E. fischeri* isolates, still finds that 90% or more of the host population may be susceptible to attack by any randomly chosen pathogen isolate. Finally, Burdon presents a range of epidemiological and genetic data from two natural host–pathogen interactions to support a general heuristic argument that envisages the evolution of gene-for-gene systems being favoured particularly in interactions in which individual host and pathogen demes are inherently unstable. In such systems, where migration is limited, life history and epidemiological considerations increase in importance and coevolution in the pathosystem as a whole may be best described by a regional process governed by a combination of drift, gene flow and various forms of selection.

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The UK Cereal Pathogen Virulence Survey

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Background

Genetic disease resistance has many advantages as a method of disease control in cereal crops. It is provided to the farmer at low cost, is relatively easy to manage and is free from environmental problems. The only risk associated with disease resistance is that it may be overcome through adaptation in the pathogen. New pathotypes are selected within pathogen populations in response to selection pressure exerted by the resistances in commercial cultivars and breeding lines. The risk is greatest when resistance depends on single major genes, or combinations of race-specific genes which have already been matched by virulence in the pathogen. It is therefore vital that pathogen populations should be monitored closely for changes in virulence. Recognition of this led to the formation of the Physiologic Race Survey of Cereal Pathogens (now the United Kingdom Cereal Pathogen Virulence Survey, UKCPVS) in 1967, following an unexpected epidemic of yellow rust (*Puccinia striiformis*) in the previously resistant wheat cultivar Rothwell Perdix.

The main objective of the UKCPVS has always been the early detection of new virulence, in order to prevent widespread epidemics. Secondary objectives include monitoring changes in the frequency of individual virulences and virulence combinations, determining the effects of changes in cultivars on pathogen populations and devising cultivar diversification schemes for use by farmers. The survey has a significant impact on the deployment of resistance genes, both by plant breeders and farmers. At the breeding stage, decisions on how best to utilize different sources of resistance can only be made with

knowledge of the virulence composition of the pathogen populations, while effective screening of early generation material often depends on inoculated tests using pathotypes which represent those found in the population. On the farm, resistance genes are deployed according to the farmer's choice of cultivar, which is strongly influenced by official evaluations of disease resistance and other important characters. Official tests for disease resistance include inoculated field tests, which are dependent on the survey for relevant pathogen isolates. Cultivar choice may also be influenced by the need to diversify, in accordance with diversification schemes based on survey results.

Organization and Methods

Responsibility for virulence testing is divided between plant pathologists at the National Institute of Agricultural Botany (NIAB) in Cambridge and the Institute for Grassland and Environmental Research (IGER) in Aberystwyth. The survey is coordinated by a Chairman and Secretary and reports to an advisory committee, which includes plant pathologists, crop advisers and plant breeders in its membership, reinforcing the emphasis on its relevance to breeders and farmers. The UKCPVS committee meets annually to discuss the results of the previous season's survey and to review policy and plans for the future. Results are published in an annual report and given wide publicity in advisory information and the farming press. Funding for the survey is provided jointly by the Ministry of Agriculture (MAFF) and the Home-Grown Cereals Authority (H-GCA), with a contribution from plant breeders.

The pathogens currently covered by the survey are listed in Table 6.1. These are all specialized pathogens which exhibit variation in virulence with respect to cultivar resistances. Priorities are under constant review, including the need to extend the survey to new pathogens.

Pathogen sampling is mainly by the collection of infected leaf samples, which is targeted at cultivars with a previous history of effective resistance, or with resistances that have only recently been overcome. This maximizes the chance of detecting new virulence and tracks the increasing frequency of recent virulences. In addition, a substantial number of samples are always taken from established susceptible cultivars. Samples are collected by plant pathologists, advisers, trials officers and farmers and sent to the appropriate testing centre for virulence analysis. At the start of each season, collaborators are provided with a list of cultivars to be targeted, together with instructions for packaging and posting. Although leaf sampling is the sole method for most pathogens, airborne spore populations of the powdery mildews are also sampled using static seedling nurseries exposed on the rooftops of high buildings, to give an indication of the virulence of the airborne population.

The number of isolates of each pathogen tested varies between pathogens and years (Table 6.2), depending on the incidence and severity of the disease

Table 6.1. Pathogens surveyed by the UKCPVS.

	Testing centre
Pathogens of wheat	
<i>Erysiphe graminis</i> (powdery mildew)	NIAB, Cambridge
<i>Puccinia striiformis</i> (yellow rust)	NIAB, Cambridge
<i>Puccinia recondita</i> (brown rust)	IGER, Aberystwyth
Pathogens of barley	
<i>Erysiphe graminis</i> (powdery mildew)	NIAB, Cambridge
<i>Puccinia striiformis</i> (yellow rust)	NIAB, Cambridge
<i>Puccinia hordei</i> (brown rust)	IGER, Aberystwyth
<i>Rhynchosporium secalis</i> (leaf blotch)	IGER, Aberystwyth
<i>Pyrenophora teres</i> (net blotch)	IGER, Aberystwyth
Pathogens of oats	
<i>Erysiphe graminis</i> (powdery mildew)	IGER, Aberystwyth
<i>Puccinia coronata</i> (crown rust)	IGER, Aberystwyth

Table 6.2. Numbers of isolates of each pathogen tested by the UKCPVS between 1989 and 1994.

	1989	1990	1991	1992	1993	1994
Pathogens of wheat						
<i>Erysiphe graminis</i>	133	525	529	194	356	347
<i>Puccinia striiformis</i>	156	67	42	77	63	68
<i>Puccinia recondita</i>	12	51	19	17	53	39
Pathogens of barley						
<i>Erysiphe graminis</i>	297	482	780	462	628	539
<i>Puccinia striiformis</i>	4	1	1	2	1	1
<i>Puccinia hordei</i>	73	49	53	77	18	12
<i>Rhynchosporium secalis</i>	13	13	50	30	69	67
<i>Pyrenophora teres</i>	14	3	15	46	7	35
Pathogens of oats						
<i>Erysiphe graminis</i>	26	15	37	42	35	32
<i>Puccinia coronata</i>	2	13	9	1	26	25

and the capacity of the testing systems. For example, powdery mildew is widespread throughout the UK in most years with no limit to the number of samples that can be obtained. In contrast, yellow rust of wheat occurs spasmodically and samples are more plentiful in epidemic years. The detached leaf system used for powdery mildew virulence tests allows relatively large numbers of isolates to be processed compared with the intact seedling methods used for most other pathogens.

Testing techniques vary between pathogens, but all are based on the reactions of differential cultivars to inoculation with the isolate being tested. Differentials possess identified specific resistance genes or resistances which are unidentified, but relevant to current cultivars and breeding programmes. Virulence tests are performed on seedlings or detached seedling leaves, to detect virulence for specific resistances which are effective at all host plant growth stages, and on adult plants, to detect virulence for resistances which are effective only at adult plant growth stages. Seedling tests are usually conducted under controlled environment conditions, as some specific resistances are known to be sensitive to environmental factors such as temperature and light intensity. Adult plant tests may be made in the field, in polythene tunnels or in controlled environment growth rooms.

Results

Early detection of virulence

By using targeted sampling, followed rapidly by virulence analysis, the survey frequently detects new virulence a year or more before it might be able to create widespread disease control problems. This allows time for appropriate action to be taken, for example alerting cultivar testing authorities and breeders, supplying isolates of new pathotypes for cultivar evaluation and issuing information through the farming press.

The example in Fig. 6.1 illustrates the detection and subsequent development of virulence for the barley powdery mildew resistance Mla13, from its first identification on the cultivar Pipkin in 1986. Before 1986, the resistance of Pipkin was fully effective against the UK mildew population, as reflected by its resistance rating of 9, the maximum point of the scale (Anon., 1986). In 1986, isolates virulent on cultivars with Mla13 resistance were obtained from the field for the first time (Wolfe *et al.*, 1987). However, in official cultivar trials, carried out by the National Institute of Agricultural Botany (NIAB) throughout the UK, only traces of mildew infection were recorded on Pipkin, and its resistance rating remained unchanged. It was not until 1988 that significant levels of mildew were detected on the cultivar in two NIAB trials and this, taken in conjunction with the UKCPVS evidence of increasing virulence frequency, prompted the reduction of the resistance rating to a figure of 4, thereby giving early warning of an impending mildew problem with this and other Mla13 cultivars. By 1989 virulence for Mla13 had become widespread in the field and Pipkin proved to be very susceptible, with a rating of only 2.

Figure 6.2 provides a similar example from yellow rust of wheat. During the 1980s extensive use was made in wheat breeding programmes of the 1B:1R translocation derived from rye, with its associated yellow rust

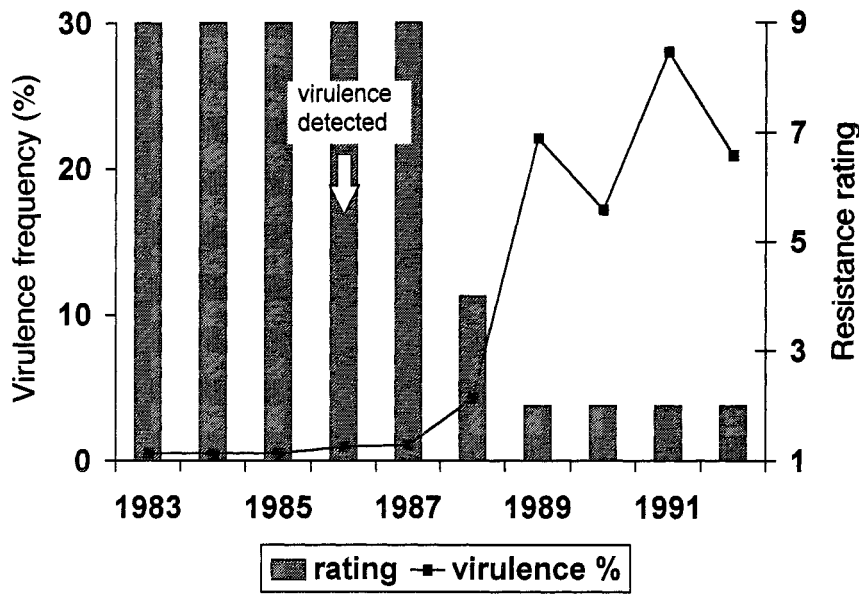


Fig. 6.1. Changes in the mildew resistance rating of the barley cultivar Pipkin following detection of virulence for Mla13.

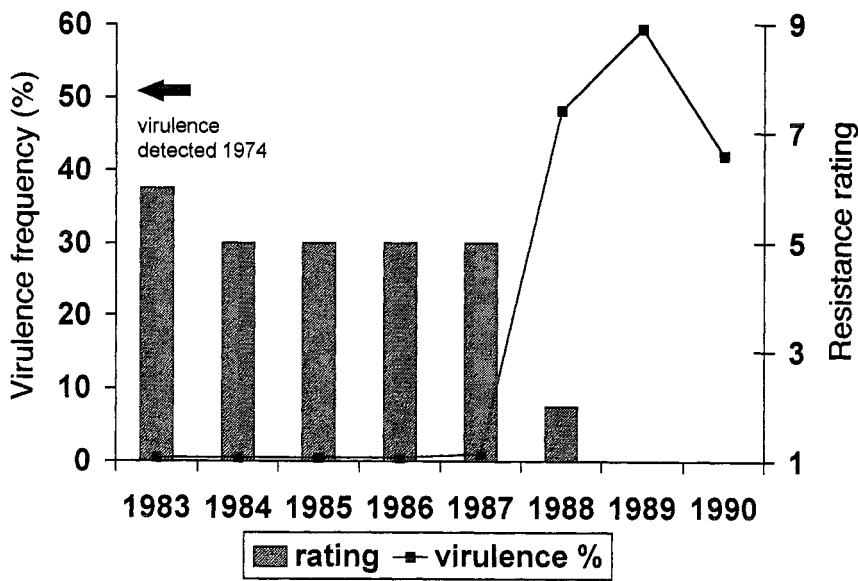


Fig. 6.2. Changes in the yellow rust resistance rating of the wheat cultivar Sleafjner following detection of virulence for Yr9.

resistance WYR9 (yellow rust resistance gene *Yr9*). Slejpner was the first commercially successful WYR9 cultivar, a number of earlier cultivars having been rapidly withdrawn because of their susceptibility to yellow rust. Virulence for WYR9 was first detected by the UKCPVS in 1974, but subsequently was recorded at only very low frequencies. Slejpner entered official cultivar trials in 1983, when preliminary inoculated tests indicated that the cultivar was susceptible, with an intermediate resistance rating of 6, falling to 5. In 1985, UKCPVS tests of new isolates indicated that Slejpner was more susceptible than its initial ratings had suggested and a warning was given that the cultivar could become a risk if widely grown (Bayles *et al.*, 1986). Two years later this prediction was fulfilled when the cultivar became severely infected in the field and its resistance rating had to be reduced to 2.

Relationship between cultivar resistance and pathogen virulence

The way in which pathogen virulence frequencies change in response to host cultivar resistances is often described as the 'boom and bust cycle' (Priestley, 1978; Brown, 1995). At the start of this theoretical cycle, the frequencies of both the newly introduced resistance and the corresponding virulence are low, so that the resistance is effective and the cultivar possessing it resistant. As the cultivar is more widely grown, the frequency of the corresponding virulence increases, the cultivar becomes susceptible on a wide scale and an epidemic ensues. As a result, the cultivar loses popularity with farmers and its acreage declines, followed by a decline in frequency of the corresponding virulence.

If this theoretical cycle occurs in practice, it should be possible not only to predict changes in virulence from changes in cultivar popularity, but also to manipulate virulence frequencies by cultivar deployment, for example the removal and reintroduction of a specific resistance. It is therefore important to establish first whether, and at what rate, the frequency of a virulence increases as cultivars with the corresponding resistance increase, and second, whether, and at what rate, the frequency of the same virulence declines as these cultivars disappear from use. These questions are addressed for wheat yellow rust and for barley mildew in diagrams showing the relationships between the frequencies of host cultivar resistance and pathogen virulence (Figs 6.3, 6.4, and 6.5).

Virulence for the wheat yellow rust resistance WYR9 was first detected in 1974, when the WYR9 cultivar Clement was still in trials in the UK, before its commercial release (Fig. 6.3). The cultivar proved to be highly susceptible and was rapidly withdrawn. A second WYR9 cultivar was briefly recommended by the NIAB between 1983 and 1984, but an increase in the frequency of the corresponding virulence exposed its poor resistance and led to its withdrawal.

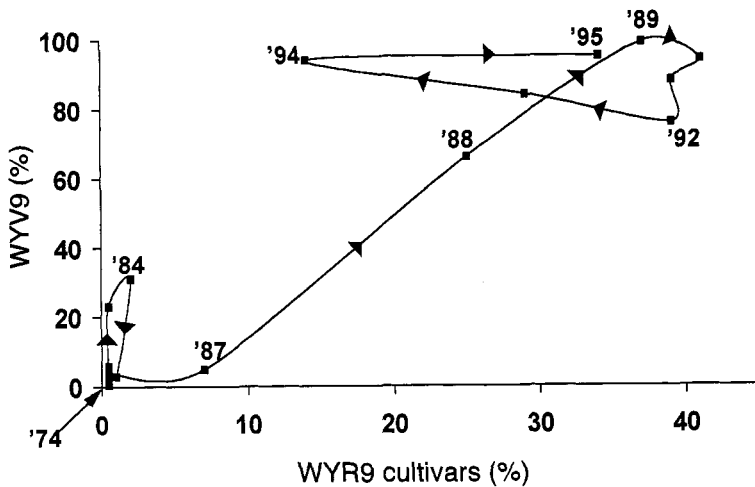


Fig. 6.3. Relationship between the acreage of wheat cultivars possessing the yellow rust resistance WYR9 and the frequency of corresponding virulence in the pathogen population.

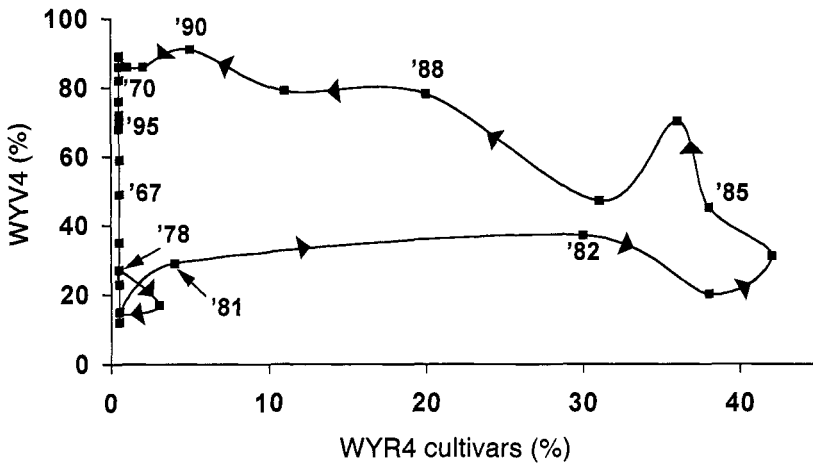


Fig. 6.4. Relationship between the acreage of wheat cultivars possessing the yellow rust resistance WYR4 and the frequency of corresponding virulence in the pathogen population.

The next significant WYR9 cultivar, Slejpner, achieved some 7% of the national acreage in 1987 before WYV9 was detected. In this instance, however, the cultivar was not withdrawn because its agronomic advantages, in particular its high yield, outweighed its susceptibility to yellow rust in the view of growers. The acreage of Slejpner and other WYR9 cultivars increased to over 40% by 1990, with a corresponding increase in the frequency of WYV9 to

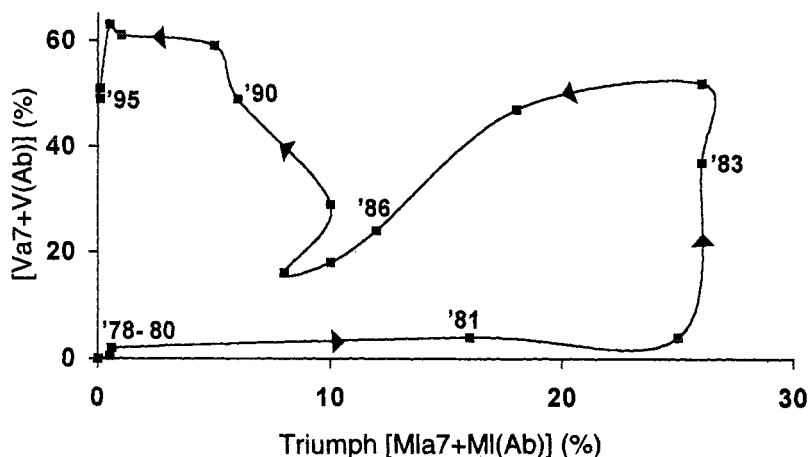


Fig. 6.5. Relationship between the acreage of the barley cultivar Triumph, possessing the mildew resistances Mla7 + Ml(Ab), and the frequency of corresponding virulence in the pathogen population.

nearly 100%. Although this was followed by a marked reduction in the acreage of WYR9 cultivars, there was no associated reduction in WYV9, which remained fixed in the pathogen population at a level of around 90%.

A second example from wheat yellow rust is that of the resistance WYR4. Virulence for WYR4 was already present at a frequency of 50% when the survey started in 1967 (Fig. 6.4). Although no WYR4 cultivars were being grown at this time, the resistance had been used in the 1950s and early 1960s and the corresponding virulence had remained in the population, where its frequency fluctuated, falling to 15% in 1980. During the following 4 years, the acreage of the cultivar Avalon, which possessed WYR4, increased to over 40%, with little response from the pathogen. This is in marked contrast to the WYR9/WYV9 relationship, where a comparable increase in acreage of cultivars was associated with an increase in virulence frequency from almost nil to nearly 100%. A probable explanation is that the WYR9 cultivars were substantially more susceptible than the WYR4 cultivar, which had a moderate degree of background resistance and rarely suffered severe infections in the field. It was not until the acreage of WYR4 started to decline during the mid- to late 1980s that the frequency of WYV4 increased, reaching about 90% in 1990. The increase in WYV4 at this stage appears to have been due to 'hitchhiking', with WYV4 being selected indirectly because of its presence in complex pathotypes with virulences which were strongly selected by other cultivars. This pattern runs contrary to the expectations of the theoretical boom and bust cycle.

An example from barley mildew is given in Fig. 6.5, which shows the relationship between the acreage of the cultivar Triumph, carrying the

resistances *Mla7* + *MI(Ab)*, and the frequency of corresponding virulence. Virulence for *Triumph* was first detected in 1978 (Wolfe and Slater, 1979), but remained at a very low level in the pathogen population until the cultivar occupied about 25% of the acreage in 1983. Between 1984 and 1988, the acreage of *Triumph* decreased and the corresponding virulence started to decline, in what appeared to be the beginning of the 'bust' phase of the classic cycle. However, over the next 7 years, the frequency of virulence for *Mla7* + *MI(Ab)* rose steeply, owing not to an increase in cultivars possessing the combined resistance, but to cultivars with either *Mla7* or *MI(Ab)* in their resistance complement.

Figure 6.6 shows the frequency of the most common pathotypes in the yellow rust population between 1996 and 1993. There has been a clear trend towards increased complexity, with pathotypes generally carrying a greater number of specific virulences than are needed to match the resistance of any individual cultivar (Bayles, 1988,1992). Between 1987 and 1993, four common pathotypes possessed WYV9, three of which also possessed WYV4. Selection for WYV9, which was strong during this period, would have resulted therefore in indirect selection for WYV4, explaining the high frequency of this apparently unnecessary virulence in the pathogen population.

Trends towards increasingly complex pathotypes have been noted in populations of other pathogens. Isolates carrying more than five virulences

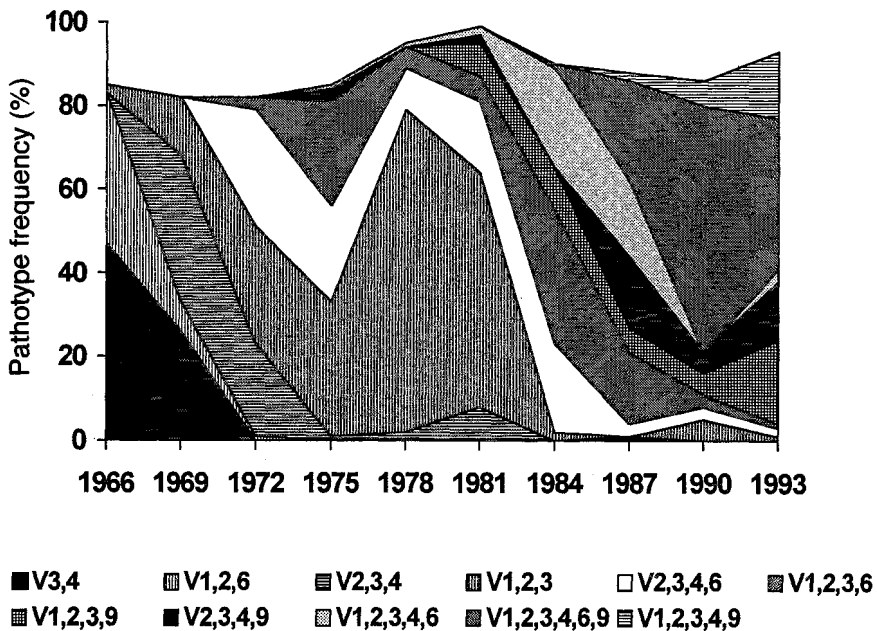


Fig. 6.6. Frequency of the most common virulence combinations detected in the wheat yellow rust population since 1966 (3-year means starting in years shown).

were common in barley mildew populations in 1992 and 1994, although there had been a slight reduction in complexity in the intervening year (Mitchell and Slater, 1995). The complexity of the wheat mildew population in 1994 meant that 25% of the population was capable of infecting 14 out of the 17 winter wheat cultivars on the Recommended List (Slater and Mitchell, 1995). Surveys of brown rust of wheat also indicate some increase in pathotype complexity between 1988 and 1994, although certain simple types still remained common (Jones and Clifford, 1995).

Geographical variation in virulence frequency

The rate at which a new virulence becomes distributed throughout the UK, whether by spread from initial sources or by many independent occurrences, has important consequences for cultivar deployment. If new virulences spread slowly, a resistance which has been overcome in one region, may continue to offer effective protection in others for a period of time. If, however, spread is rapid, resistances overcome in one region will immediately be at risk in all others.

Barley mildew occurs widely throughout the UK in all seasons and has highly mobile spores, characteristics which might be expected to lead to rapid distribution of new pathotypes. There has been little evidence of regional differences in virulence frequencies except, occasionally, between England and Scotland. Such differences are usually related to the cultivars being grown in these regions. For example, the slower increase in virulence for Mla7, Ml(Ab) in Scotland was attributed to the slower uptake of the host cultivar Triumph in Scotland (Wolfe *et al.*, 1985), where it was less popular because of its late maturity. In 1991, there was an indication that virulence for Mla13 was more frequent in Scotland, where cultivars with the Mla13 resistance were more popular, although this difference had disappeared by 1992, with the increased use of Mla13 cultivars in England and Wales (Mitchell and Slater, 1993).

In contrast to mildew, yellow rust of wheat has a limited geographical distribution, being largely restricted to eastern areas of England and Scotland in most seasons. It only occurs severely outside these areas one year in every three or four. The spread of new virulences might therefore be expected to be less rapid. Figure 6.7 shows the frequency of the virulence combination WYV6,9 in different areas of the UK in the 5 years following its first detection in 1988 in Scotland and North-East England. Cultivars with the resistance WYR6,9 were at an early stage of commercialization throughout the UK at this time, being grown on less than 1% of the acreage, with no obvious regional bias. By the following year, 1989, WYV6,9 was approaching 100% in Scotland and the North-East and was already present at over 60% frequency in the east midlands and East Anglia and at a slightly lower frequency in other regions of the UK. By 1992, the virulence combination had become more

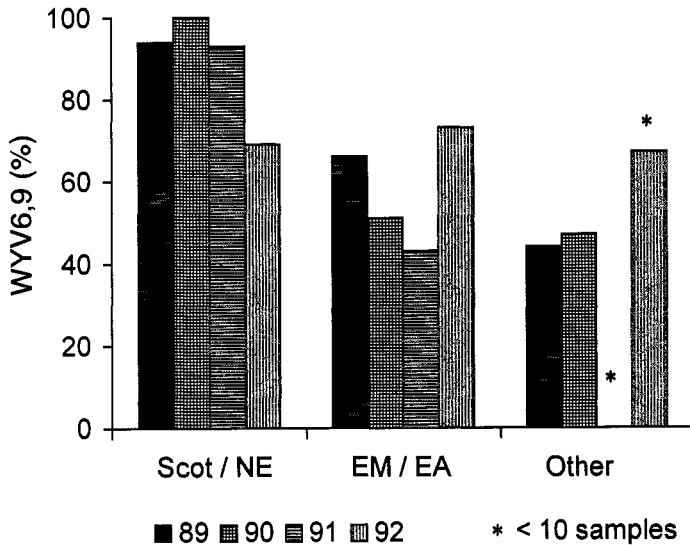


Fig. 6.7. Frequency of the wheat yellow rust virulence combination WYV6,9 in three regions of the UK during the 4 years following its detection in 1988. (Scot/NE = Scotland and North-East England; EM/EA = East Midlands and East Anglia; Other = all other regions of the UK.)

evenly distributed across England and Wales, demonstrating the potential for rapid increase of a new virulence, even in regions of the country where there is a low risk of yellow rust and relatively few outbreaks of the disease.

It appears that although regional differences in pathogen virulence frequency may occur occasionally, they are likely to be short lived and of no practical significance for cultivar deployment. The same cultivars tend to be grown widely throughout the UK and although there may be some regional differentiation, this is not clear enough to maintain distinct differences in pathogen virulence.

Impact on plant breeding and cultivar evaluation

It has always been a major aim of the survey to contribute to the improvement of disease resistance by supporting plant breeding and cultivar evaluation. This has been achieved primarily by ensuring that critical pathogen isolates are available for use in inoculated tests of breeding material and of cultivars which are candidates for official recommendation. Resources are saved at the breeding stage, as breeders are able to reassess their strategies as soon as a particular resistance is overcome. Similarly, cultivar testing organizations are able to determine the specific resistances carried by candidate varieties and assess

their background resistance once these specific resistances are matched by the pathogen. The 1 to 9 disease resistance ratings issued by the NIAB utilize this information and describe the potential susceptibility of cultivars when challenged by virulent pathotypes.

A recent example is the impact of the survey on wheat cultivars possessing the yellow rust resistance gene *Yr17* (WYR17). During the late 1980s and early 1990s, widespread use was made of this gene in wheat breeding programmes. *Rendezvous*, the first UK cultivar to carry WYR17 was recommended by the NIAB in 1987. This was followed by a number of more successful cultivars, amongst them *Hussar* in 1992 and *Brigadier* in 1993 and, by 1995, it was estimated that over half the entries in national list trials had WYR17 in their pedigree. Virulence for WYR17 was not detected until 1995, when initial tests using the new pathotype indicated that cultivars carrying the resistance differed widely in their levels of background resistance. The immediate task will now be to identify and discard those cultivars and lines with inadequate background resistance, using definitive pathogen isolates from the Survey. In the longer term, there is likely to be a move away from the use of WYR17 to alternative sources of resistance.

Cultivar diversification schemes

The aim of cultivar diversification is to limit the spread of disease by growing cultivars with different specific resistances, either in neighbouring fields or in cultivar mixtures. In the UK, field-to-field diversification is the more usual choice of farmers and mixtures have achieved little popularity. The UKCPVS publishes diversification schemes, which take account both of the specific resistances of cultivars and the virulence composition of the pathogen population (Priestley and Bayles, 1980). The underlying principle is that disease is unlikely to spread between cultivars possessing different specific resistances, because spores generated on one are largely avirulent on the other. However, where combined virulence for two or more specific resistances is common, the risk of disease spread between cultivars with these different specific resistances may be as great as between cultivars with the same specific resistance and diversification is ineffective. Evidence that diversification can be effective in reducing the spread of disease has been summarized by Priestley and Bayles (1982). Field-to-field diversification can be regarded as an insurance measure, since it reduces the likelihood of a farmer's entire wheat or barley acreage being affected by disease at the same time.

Schemes are currently available for mildew of barley, yellow rust of wheat (Box 6.1) and brown rust of wheat. A scheme for mildew of wheat was discontinued in 1990, its usefulness having been severely restricted by the limited range of specific resistances in current cultivars and the increasing complexity of the mildew population.

Box 6.1. Varietal diversification scheme to reduce spread of yellow rust in wheat (1996).

Severe infections may result if yellow rust spreads between varieties which are susceptible to the same races of the pathogen. This risk is reduced if varieties with high levels of resistance are grown. The spread of disease can be limited further by growing different varieties in neighbouring fields, provided that they are not susceptible to the same races of yellow rust. The Diversification Scheme should be used to choose varieties to grow next to one another.

Choosing varieties to grow together

1. Select first-choice variety and locate its Diversification Group (DG). (S) = spring variety.
2. Find this DG under 'Chosen DG' down the left-hand side of the table.
3. Read across the table to find the risk of disease spread with each companion DG.
 - + = low risk of spread of yellow rust
 - Y = high risk of spread of yellow rust
 - y = moderate risk of spread of yellow rust
4. Wherever possible choose combinations of varieties marked '+'. A combination marked 'y' is a lesser risk than one marked 'Y'.

DG1	DG1 (cont)	DG2	DG7 (cont)	DG0
Buster	Magellan	Haven	Spark	Genesis
Cadenza	Mercia	Rialto		Soissons
Caxton	Raleigh		DG9	Avans (S)
Charger	Axona (S)	DG3	Beaufort	Baldus (S)
Dynamo	Chablis (S)	Riband	Brigadier	Canon (S)
Encore	Imp (S)	DG7	Chianti	Minx (S)
Flame	Shiraz (S)	Consort	Hussar	Palermo (S)
Hunter		Hereward	Reaper	Promessa (S)

Chosen DG	Companion DG					
	1	2	3	7	9	0
1	+	+	+	+	+	+
2	+	Y	y	y	y	Y
3	+	y	Y	y	+	Y
7	+	y	y	Y	+	Y
9	+	y	+	+	Y	Y
0	+	Y	Y	Y	Y	Y

Note: Varieties in DG1 have good resistance to yellow rust spreading from any other variety and can therefore be used to diversify with varieties in any DG, including others in DG1. Varieties in DG0 are susceptible to yellow rust spreading from any variety and therefore do not contribute to diversification.

Conclusions

Sustained improvement of disease resistance in cereal cultivars, by breeding and evaluation, can only be achieved against a background of continual pathogen virulence monitoring, designed to detect new virulences and follow changes in the frequency of virulences and their combinations. Changes in virulence are largely unpredictable. Although it is common for resistance based on one or two major genes to be overcome over a period of years, the timing of the first appearance of virulence is variable. Virulence may not be detected until cultivars possessing the corresponding resistance have become widely grown, but it is equally likely to emerge before they reach commercialization. Virulence frequency usually rises as the acreage of cultivars with corresponding resistance increases, particularly if the specific resistance is in a highly susceptible background. However, it rarely returns to low levels once the resistance disappears from use and may either remain stable or even increase in frequency as a result of 'hitch-hiking'. This deviation from the theoretical 'boom and bust' model makes it unlikely that resistances can usefully be reintroduced once overcome. The unpredictable nature of the response of pathogen populations to cultivar resistances reinforces the need for long-term monitoring.

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Adaptation of Powdery Mildew Populations to Cereal Varieties in Relation to Durable and Non-durable Resistance

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Gene-for-Gene and Other Resistances

Three types of resistance to powdery mildew are known in cereals. In the UK, the kind which has contributed most to durable control of mildew is non-race-specific, partial resistance (Shaner, 1973; Asher and Thomas, 1983; Jones and Davies, 1985), which forms the basis of mildew resistance in most of the currently important winter wheat and winter barley varieties. Secondly, the *mlo* gene has provided durable mildew resistance in spring barley breeding for over 20 years (Jørgensen, 1992).

The third type is race-specific resistance, based on the gene-for-gene relationship, which is the best understood of the three types in terms of mechanisms and genetics. Many gene-for-gene resistances have been used in plant breeding, but they have contributed remarkably little to long-term, effective disease control because populations of the mildew pathogen, *Erysiphe graminis*, have adapted more or less rapidly to varieties with these resistances. (In this chapter, we use the term 'gene-for-gene resistance' in preference to other terms, such as 'major gene resistance', which includes single genes which do not follow the gene-for-gene mechanism, or 'race-specific' resistance, which includes polygenic resistances which have some race-specificity.)

In this chapter, we review briefly the use of gene-for-gene mildew resistances in cereals in Europe and summarize the process, as it is presently understood, by which *E. graminis* adapts to cereal varieties. The largest part of this review discusses how knowledge of the process of adaptation is developing, the significant gaps in this knowledge and the technical developments that will

be required to advance our understanding. Finally, we discuss the extent to which knowledge about adaptation to gene-for-gene resistance has implications for other types of cereal mildew resistance.

The Use of Gene-for-Gene Resistances in Cereal Breeding

In barley, most of the gene-for-gene resistances which have been used in European breeding programmes are located on the short arm of chromosome 5 (= 1H). These include *Mla1*, *Mla3*, *Mla6*, *Mla7*, *Mla9*, *Mla12* and *Mla13* at the *Mla* locus, *Mlat*, *Mlk1* and *Mlra*. Important genes located elsewhere include *Mlg* and *Ml(CP)* (chromosome 4), *Mlh* (chr. 6) and *MILa* (chr. 2). Another important gene, *Ml(Ab)*, has not so far been located. There are many other resistance genes which are either present in Asian barleys or have been identified in landraces or wild barley accessions (Jørgensen, 1993, 1994).

Fewer race-specific resistance genes have either been used in breeding for wheat mildew resistance in Europe or are currently being introduced. They include two genes, *Pm3b* and *Pm3d*, at the *Pm3* locus (Zeller *et al.*, 1993), which is probably homologous with *Mla* (Hartl *et al.*, 1993). Two other genes, *Pm8* (Bennett, 1984) and *Pm17* (Heun *et al.*, 1990), are on short arms of 1R chromosomes of rye, translocated into wheat, and may also be homologous with *Pm3* and *Mla*. Other important race-specific genes in European varieties are *Pm1* (chr. 7A), *Pm2* (chr. 5D), *Pm4b* (chr. 2A), *pm5* (chr. 7B), and *Pm6* (chr. 2B); while *Pm3a*, *Pm3c*, *Pm3f* (1A), *Pm7* (4B), *Pm9* (7A) and *Mld* (4B) have been used in a few varieties in Europe or elsewhere (Bennett, 1984; McIntosh *et al.*, 1995).

In both barley and wheat, gene-for-gene resistances have only provided temporary control of mildew, lasting for 2 to 5 years. Figure 7.1 summarizes the 'breakdown' of some mildew resistances in UK barley varieties. The process by which *E. graminis* f. sp. *hordei* (barley powdery mildew) adapts to new varieties of barley has been reviewed recently (Brown, 1994); it is likely that a similar process applies in wheat. It is proposed that adaptation happens in the following steps:

1. When a new resistance is introduced, it is effective if the frequency of the matching virulence is low; one or very few pathogen clones multiply on varieties with the newly introduced resistance.
2. These virulent clones are dispersed to other fields of varieties with the same resistance, often over hundreds of kilometres.
3. The rapid multiplication of a small number of clones leads to hitch-hiking selection, in which virulences which are not themselves selected increase in frequency because they are carried by the selected clones. This causes rapid fluctuations in the frequencies of the unselected virulences.

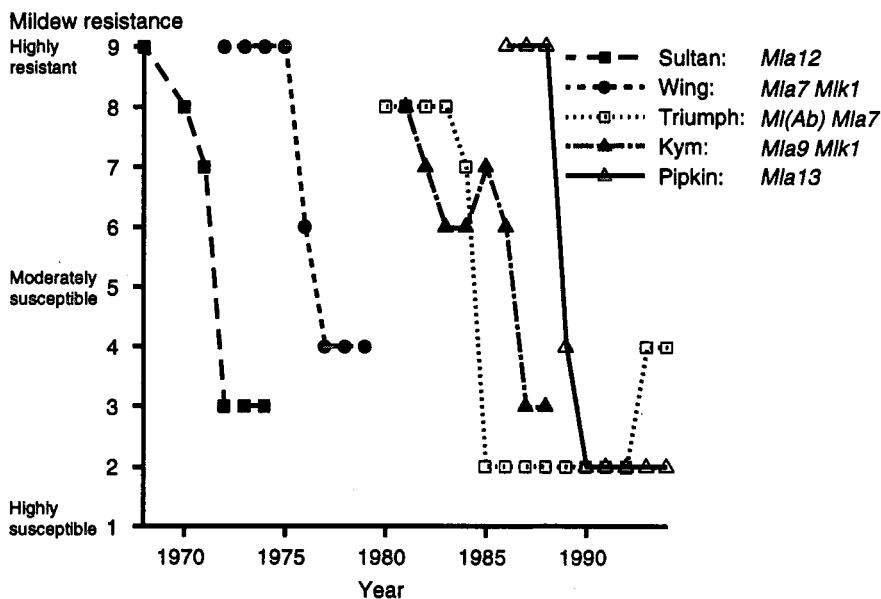


Fig. 7.1. The 'breakdown' of some barley powdery mildew resistance genes. The varieties named were the first in the UK to carry the resistance genes *Mla12*, *Mla7* + *Mlk1*, *Ml(Ab)*, *Mla9* or *Mla13*; Triumph and Kym also had *Mla7* and *Mlk1* respectively. The powdery mildew resistance ratings are those given in *Recommended Varieties of Cereals* by the National Institute of Agricultural Botany. 9 or 8 indicate good resistance, 7 or 6 moderate resistance, 5 or 4 moderate susceptibility and 3, 2 or 1 high susceptibility.

4. Recombination and further mutation increase the diversity of clones carrying the new virulence.
5. Selection among diverse clones increases the mean fitness of the *E. graminis* population on cereal varieties.

Mutation to Virulence

The first step in the postulated process of adaptation is the multiplication of one or a very few *E. graminis* clones, carrying the matching virulence, on the new, resistant varieties. Two cases should be distinguished, one in which a variety has a gene which has not been used before, and one in which a variety has a combination of genes which have been used previously, such that they are effective together but not separately. We consider the former case here and the latter in the section on Hitch-Hiking Selection.

When a new, effective resistance gene is introduced, the frequency of the matching virulence rises from a very low level. Essentially, a mutant is selected

from the *E. graminis* population (although, given that natural selection acts on existing variation, the mutant may have existed in the population for some time). Such a process appears to have happened at least twice in barley mildew in the British Isles in the 1980s, once on Triumph (*MI(Ab)* + *Mla7*) (Brown and Wolfe, 1990; Brown *et al.*, 1990) and once on a group of varieties carrying *Mla13* (Brown *et al.*, 1991; Wolfe *et al.*, 1992).

The mechanism of most gene-for-gene resistance in barley is based on the hypersensitive response, such that infected epidermal cells, and, in some interactions, surrounding epidermal and mesophyll cells, die when the pathogen reaches a particular stage of development. The time at which cell death occurs and the extent of the cell death response are correlated; for instance, interactions involving *Mla1* or *Mla6* occur earlier than those involving *Mla3* or *Mla7* and result in fewer host cells dying (Boyd *et al.*, 1995). *MLg*, however, also has a second, unknown mechanism, which inhibits pathogen development before hypersensitive cell death occurs (Görg *et al.*, 1993).

The standard gene-for-gene model is based on the assumption that one resistance gene interacts with one avirulence gene. This model does indeed apply to avirulences matching most powdery mildew resistance genes in wheat and barley (Hiura, 1964; Moseman, 1966; Jørgensen, 1988; Christiansen and Giese, 1990; Brown and Simpson, 1994; Brown and Jessop, 1995; Jensen *et al.*, 1995; Brown *et al.*, 1996). In these cases, one would expect that a single mutation from avirulence to virulence may be all that is necessary for the pathogen to overcome the resistance gene.

However, there are some notable exceptions to this rule, the best studied of which is avirulence matching *Mla13*. Brown and Simpson (1994) and Jensen *et al.* (1995), studying different crosses of *E. graminis* f. sp. *hordei* isolates, found high frequencies of avirulent progeny. Both groups postulated that two avirulence genes matched this resistance, because the segregation ratio of avirulent:virulent was not significantly different from 3:1 (note that *E. graminis* is haploid); a progeny isolate would only be virulent if it lacked both avirulence functions. Test crosses have shown that two *Avr_{a13}* avirulence genes, matching the *Mla13* resistance, do indeed segregate in CC52 × DH14 (Caffier *et al.*, 1996a).

Segregation data suggest that several other avirulence phenotypes may involve interactions between several genes, although the genetic hypotheses have not been tested. In CC151 × DH14, segregation of avirulence towards *Mla6* is consistent with there being two matching genes, *Avr_{a6-1}* and *Avr_{a6-2}* (Brown *et al.*, 1996). *Mla6* is closely linked to another gene, *Mla14* (Mahadevappa *et al.*, 1994), but infection type data indicate that neither of the *Avr_{a6}* genes match *Mla14*. In most crosses, only one avirulence gene matches *MLk1*, derived from Kwan or Hordeum 1063 (Hiura, 1964; Moseman, 1966; Jørgensen, 1988; Christiansen and Giese, 1990; Brown and Jessop, 1995; Jensen *et al.*, 1995). However, again in CC151 × DH14, only one gene, *Avr_{k1}* matched *MLk1* derived from Hordeum 1063, but two avirulence genes, *Avr_{k1}*

and *Avr_{P17}*, matched the *Mlk1* resistance in Pallas-17, derived from Monte Cristo (Brown *et al.*, 1996). The most complex avirulence is that matching *Mla7*. Brown and Jessop (1995) found two genes, *Avr_{a7-1}* and *Avr_{a7-2}*, matching *Mla7* in all four of the sources from which it has been introduced into barley breeding. Jensen *et al.* (1995), however, found one avirulence gene matching *Mla7* in three of the four sources of *Mla7* (the fourth source, Triumph, was not tested), and a total of four more genes matching *Mla7* in one or other of these three sources.

There are essentially two hypotheses to account for data such as these. One is that several resistance genes are clustered at both the *Mla* and *Mlk* loci, in a manner similar to that of the *M* rust-resistance locus in flax or the *Rp1* rust-resistance locus in maize (Pryor and Ellis, 1993). The occurrence of recombination between *Mla6* and *Mla14* (Mahadevappa *et al.*, 1994) suggests that this is indeed a possibility. In this scenario, Monte Cristo would have a resistance gene, closely linked to *Mlk1* but not carried by Hordeum 1063, while there would be a third gene linked to *Mla6* and *Mla14*. Also, all four sources of *Mla7* may have a second gene, closely linked to *Mla7* and matched by *Avr_{a7-2}*, while the three sources studied by Jensen *et al.* (1995) may have additional genes linked to *Mla7*.

Alternatively, it could be that two avirulence genes match one resistance gene; in *Arabidopsis thaliana*, the *RPS3/RPM1* resistance gene matches two avirulences in *Pseudomonas syringae* (Bisgrove *et al.*, 1994). This hypothesis is not inconsistent with the essential physiological principle of the gene-for-gene relationship. It could be that more than one pathogen molecule interacts with a single resistance gene product to induce a hypersensitive response. It will only become possible to compare the two hypotheses rigorously once the matching host and pathogen genes have been cloned, and functional studies of the interaction between resistance and avirulence gene products are under way.

Regardless of the mechanism by which two avirulence genes match one source of resistance, it is clear that in several cases, more than one virulence mutation must have been carried by the *E. graminis* f. sp. *hordei* clones which first overcame some resistance genes. One consequence of this relates to the proposition that durable resistance could be achieved by 'gene pyramiding' – introducing several resistances simultaneously in a single new variety (Flor, 1955). We need only consider the case of Wing spring barley (Fig. 7.1) to see the inadequacy of pyramiding as a strategy; at least three avirulence functions – probably more – had to be lost before an *E. graminis* f. sp. *hordei* clone could overcome Wing's resistance, conferred by *Mla7* + *Mlk1*, yet this combination eventually became as ineffective as any other gene-for-gene resistance. A second consequence is that models of the population genetics of *E. graminis* (Østergård and Hovmøller, 1991; Hovmøller *et al.*, 1993; Brown, 1995a) may be flawed if they are based on a strictly one-for-one gene-for-gene relationship.

Dispersal of Virulent Clones

Once a virulent clone has become established on a variety with a new resistance, it may be dispersed by the wind and become established in other fields, either of the same variety or of other varieties with the same resistance. Two years after the *Mla13* gene was introduced in barley varieties in 1986, three clones of *E. graminis* f. sp. *hordei* were found in locations several hundred kilometres apart in the British Isles (Brown *et al.*, 1991); the most common of these may have originated in the former Czechoslovakia, where *Mla13* was first used (Wolfe *et al.*, 1992).

The introduction of a new resistance gene into a breeding programme is expensive (Lawes, 1988). While the simultaneous use of one source of resistance by many breeders in Europe is understandable from the economic point of view, it leads to a situation where the matching virulence can evolve on one variety in one part of Europe and then spread rapidly to other varieties in other regions. Conidiospores of *E. graminis* can be dispersed by the wind over long distances (Hermansen *et al.*, 1978). Although analysis of virulence phenotype frequencies suggests that established populations of *E. graminis* are dispersed in the direction of the prevailing wind, in the order of 100 km a year (Limpert, 1987), individual spores may be dispersed by winds of any direction – as in the apparent east-to-west migration of *Mla13*-virulent clones.

The frequency of migration between regions of Europe is limited, however, both geographically, by distance and by features such as the Alps, and by regional variation in the use of different resistance genes in varieties. Although clones with virulence towards recently introduced resistances may be found in many parts of Europe, there is also a diverse fraction of the population which is differentiated at the scale of, very roughly, a few hundred kilometres (Müller *et al.*, 1992; Brown, 1994).

Hitch-Hiking Selection

Selection of a clone by a new resistance in what has been termed a 'founder event' (Brown, 1995b) causes all of the alleles carried by that clone to increase in frequency, not just those that are selected. Virulences which have 'hitch-hiked' in this way include Va6 (i.e. virulence towards *Mla6*), Vk1 and V(CP) in a clone virulent on Triumph spring barley in the early 1980s (Brown and Wolfe, 1990), Va12 in a clone virulent on Klaxon and Doublet spring barley in the summer of 1986 (Brown *et al.*, 1993) and Va7, Va9 and Vk1 in clones virulent on *Mla13* barley varieties in 1988 (Brown *et al.*, 1991).

Hitch-hiking leads to rapid, unpredictable changes in frequencies of virulences and associations between virulences. The most dramatic event observed of this kind occurred in 1986 (Brown *et al.*, 1993). In June of that year, the *E. graminis* f. sp. *hordei* population was dominated by a group of clones

virulent on Triumph, many of which had also been detected in population samples in 1985. These clones carried Va7 and V(Ab), needed for virulence on Triumph; most clones had an unnecessary virulence, Vk1, not needed for successful infection of Triumph, while the most frequent clone had another unnecessary virulence, Va6. Most clones lacked Va12 and VL_a. By October 1986, a clone which had had a frequency of less than 1% in June had risen to a frequency of over 35%. This clone was virulent on two spring barleys which had first been grown on a large scale in 1986, Klaxon and Doublet, because it carried Va7 and VL_a, needed for virulence on both varieties, and Vk1, needed for virulence on Klaxon. It also had another, unnecessary virulence, Va12, and lacked Va6 and V(Ab). As a result of the rapid replacement of the Triumph-virulent clones by the Klaxon/Doublet-virulent clone, many associations between these six virulences diminished or even became reversed in sign. Furthermore, Va6, which had been maintained at a high frequency by virtue of its presence in the most important Triumph-virulent clone, despite the absence of *Mla6* from UK barley varieties, now fell in frequency because it was not carried by the Klaxon/Doublet-virulent clone.

Theoretical modelling (Brown, 1995a) suggests that this type of hitch-hiking can alter the frequency of an unnecessary virulence significantly if the rate of recombination between it and the selected virulence (the product of the frequency of sexual reproduction and the genetic recombination fraction) is less than the fraction of the host population which carries the resistance gene that selects the virulence. Hitch-hiking can also increase the frequency of an unnecessary virulence carried by the selected clone, even if the unnecessary virulence causes a loss of fitness, provided that the coefficient of selection against the unnecessary virulence is no higher than half the frequency of the selectively resistant host; again, the strength of the hitch-hiking effect depends on the frequency of recombination. These results indicate that attempts to infer the existence of selection for or against virulences (or other phenotypes), or to estimate its value, in a partly clonal pathogen, simply on the basis of phenotype frequencies in samples from populations, are unlikely to yield reliable conclusions. For example, although Grant and Archer (1983) ascribed the drop in frequency of Va6 between 1969 and 1975 to reduced fitness of isolates with the Va6 phenotype, this drop could have been caused by the hitch-hiking effect, because Va12 clones, lacking Va6, were selected by *Mla12* varieties (Wolfe, 1984).

A different type of hitch-hiking has been described by Hovmøller *et al.* (1993). This process starts from a situation in which two virulence genes are neither positively nor negatively associated in the population of a pathogen (i.e. they are in linkage equilibrium). The resistance matching one of the virulences is then introduced, causing selection for that virulence; the two virulences remain in linkage equilibrium. If the second resistance is then introduced, replacing the first resistance, the second virulence is selected. Sequential selection, caused by resistances being used in different varieties at different

times, causes negative linkage disequilibrium between the matching virulences. This could have been the reason why Va6, Va7, Va12 and VL_a, matching Mla6, Mla7, Mla12 and ML_a respectively, were generally dissociated from one another in the British *E. graminis* f. sp. *hordei* population in the 1970s (Wolfe, 1984).

Recombination and the Generation of Diversity

Sexual reproduction is important in the population genetics of *E. graminis* in two ways. First, it is directly relevant to the evolution of virulence. Once a virulence has become established in the population, new genotypes with that virulence may arise either by recombination, so that the original virulence allele becomes incorporated into new genotypes, or by further, different mutations of the avirulence gene in other clones. It will only be possible to test which of these two mechanisms has produced a new, virulent genotype once cloned avirulence genes are available.

Second, despite the occasional emergence of clones which reach high frequencies over large areas, populations of *E. graminis* f. sp. *hordei* are usually highly diverse (Wolfe *et al.*, 1992; Wolfe and McDermott, 1994). Indeed, even during a founder event, two fractions of the population can be distinguished, one consisting of a few closely related clones and one composed of many, highly diverse clones, each at a low frequency (Brown *et al.*, 1990, 1993). The two mechanisms which can increase the diversity of a partly clonal population are recombination and mutation. More attention has been paid to recombination as a means of generating diversity, because it is unlikely that the mutation rate is sufficiently high to account for the rate of diversification of *E. graminis* f. sp. *hordei* populations. Since the effective population size of *E. graminis* f. sp. *hordei* is of the order of 10^9 (Damgaard and Giese, 1996), the rate of introgression of a new mutation into the population is extremely slow (Hedrick, 1985).

E. graminis survives the summer either in the sexual phase, as cleistothecia, or in the asexual phase, as mycelium. Cleistothecia of *E. graminis* form in mid-summer, as the host plants are senescing, and hatch in autumn, to release ascospores on to seedlings of the winter crop. Also, colonies of *E. graminis* form on volunteer seedlings and produce conidiospores which infect the winter crop in the autumn. The autumn epidemic of *E. graminis* is therefore initiated by inoculum derived from the summer epidemic population by both sexual and asexual means (Brown, 1994).

In studies of the possible effect of recombination on the population genetics of *E. graminis* f. sp. *hordei*, Welz and Kranz (1987) observed greater diversity in a German population in autumn 1984 than in the summer of that year; however, a similar result was not obtained in 1985. Brown and Wolfe (1990) found that most linkage disequilibria decreased between summer and autumn in an English population in 1985. They estimated that 25% of the autumn

population was derived from ascospores formed that summer; however, the confidence interval is so broad as to render this estimate meaningless (Brown, 1994).

In diploid organisms, the frequency of recombination in partly selfing or partly apomictic populations can be estimated from the frequency of heterozygosity, since one cycle of completely sexual reproduction is expected to restore heterozygosity to Hardy–Weinberg equilibrium (Hedrick, 1985). This is obviously not possible for haploid organisms. However, sex causes two other population genetic phenomena: it tends to equalize the frequencies of the two mating types (if the organism is heterothallic), and it reduces linkage disequilibrium (D). One cycle of completely sexual reproduction restores both mating types to a frequency of 0.5 and halves D .

The frequency of recombination, x , can therefore be estimated from the extent to which the frequencies of the two mating types (m_1, m_2 ; $m_2 = 1 - m_1$) tend to 0.5 between summer and autumn. Let the values of m_1 before and after sex has occurred be m_{1B} and m_{1A} respectively. In the fraction x of the autumn population of *E. graminis* which descends sexually from the summer population, the two mating types both have frequencies of 0.5, since half the progeny of any cross inherit each mating type allele. In the fraction $1 - x$ which descended asexually, the two mating types are at the same frequencies as in the summer population. This gives

$$m_{1A} = 0.5x + m_{1B}(1-x)$$

(Fig. 7.2). Although we are only interested in the value of x , we need to estimate both m_{1B} and x (m_{1B} is a 'nuisance parameter'). A method of doing this, using profile likelihood (McCullagh and Nelder, 1989), has been developed and will be described elsewhere (J.K.M. Brown and P.M.E. Altham, unpublished).

The principal conclusion from our studies so far is that enormous numbers of individuals need to be sampled in order to estimate x accurately (Fig. 7.3). For instance, for $m_{1B} = 0.7$ and an actual frequency of sex of 0.3, simulations indicate that nearly 4000 individuals must be sampled, both before and after sex has occurred, to have a confidence interval for x of less than 0.2. The accuracy is somewhat improved if the mating type frequencies before sex are more extreme, but even then, many hundreds of samples, or even thousands, need to be collected. Estimates of x from a few hundred samples (Brown and Wolfe, 1990; Brändle *et al.*, Chapter 9 this volume) are therefore likely to be highly inaccurate. The most efficient way of testing this number of samples would probably be a dot blot system in which crude DNA extracts from many individuals are probed with sequences which differentiate the two mating types. However, such sequences are not yet available for *E. graminis*. A possible alternative might be to use a sequence closely linked to the mating type locus (Brändle *et al.*, Chapter 9 this volume), and to introduce another, fixed parameter into the model, the recombination fraction, r , between the test sequence and the mating type locus. (It is not possible to estimate both r and x from a

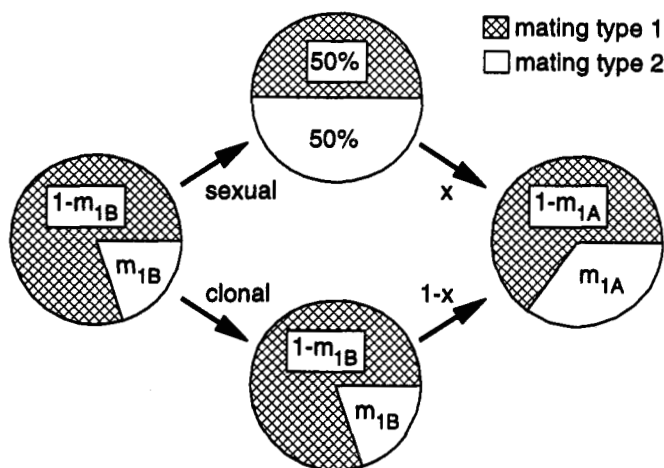


Fig. 7.2. The tendency of the frequencies of the two mating types (1 and 2) to equalize after a period during which sexual reproduction occurs. The frequency of mating type 1 before sex is m_{1B} ($= 0.2$ in this diagram), and after sex, m_{1A} ($= 0.35$). The frequency of sex is x ($= 0.5$).

single population sample since the two estimates would be wholly confounded.) This raises the philosophical question of why a value of r , determined by analysis of a relatively small number of progeny of a cross and therefore having a fairly broad confidence interval of its own, should be used as a fixed parameter to estimate the value of x from a very much larger population sample; the estimate of x would depend on the estimate of r , and the error in the latter estimate would introduce further undesirable error into the estimation of x .

Clearly, estimation of the frequency of sex in *E. graminis* is currently fraught with difficulty and the values of x obtained so far are all extremely unreliable. The value of an estimation procedure would be to allow examination of the extent to which host species, varieties, cropping systems and environmental conditions alter x , but this is not yet possible. Furthermore, attempts to estimate x may need to take account of other factors, such as selection (Brown *et al.*, 1993; Caffier *et al.*, 1996b), migration (Hovmöller *et al.*, 1993) or genetic drift (Brändle *et al.*, Chapter 9 this volume), which operate between the times that the two samples are collected.

Selection in Mildew Populations

The genetic variation in *E. graminis* populations (Brown *et al.*, 1990; Wolfe *et al.*, 1992; Wolfe and McDermott, 1994) can be acted on by natural selection to increase mean fitness on host varieties. Wolfe and Schwarzbach (1978)

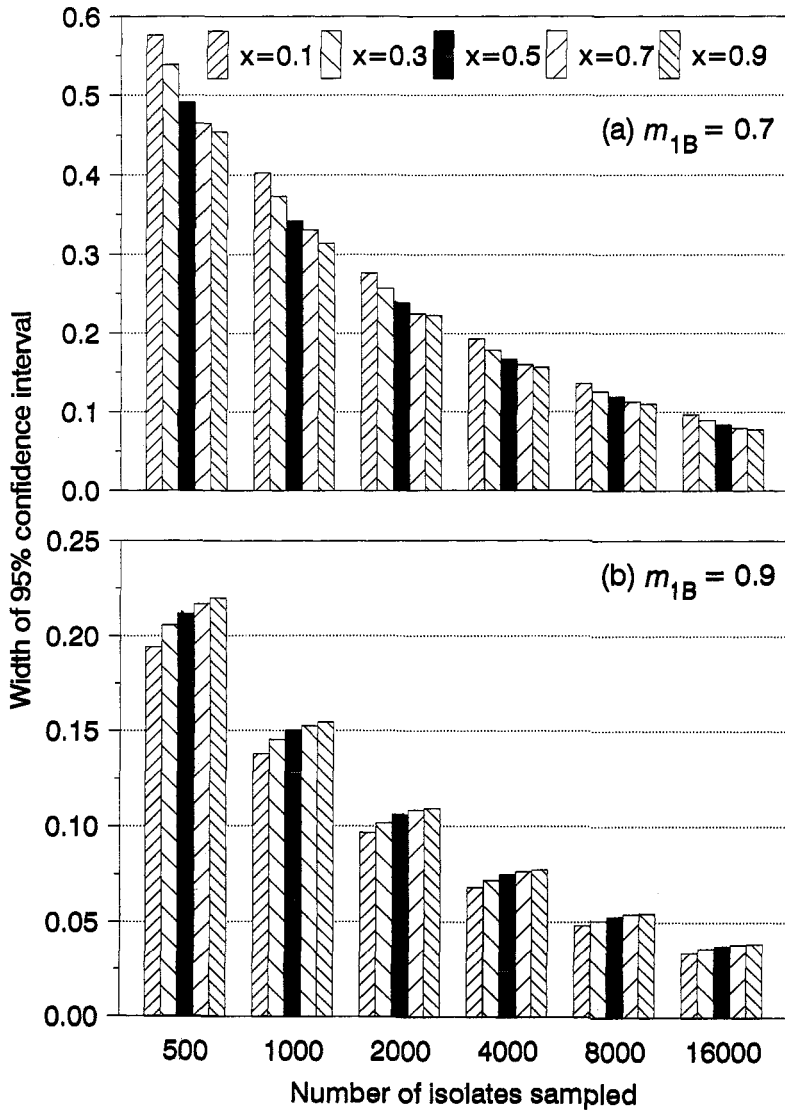


Fig. 7.3. The effects of sample size on the accuracy of estimation of the frequency of sex in a partially clonal haploid organism. Simulations used actual frequencies of sex of x and frequencies of one of the two mating types before sex of m_{1B} , as shown. The number of isolates indicates the sample sizes used both before and after the period during which sex occurs.

suggested that adaptation of a pathogen to a host variety occurs in two stages: first, the variety selects clones with all of the race-specific virulence genes that match the host's resistance genes, and subsequently, selection occurs among these clones for adaptation to the variety's 'background' resistance.

In 1995, we did a field trial to test if the second stage of this process, adaptation to the varietal background, does in fact occur. The question asked was whether or not the infection efficiency of isolates was higher on the variety from which they were sampled than on another variety. We sowed two 10 m square plots (100 m²) each of the spring barley varieties Golden Promise (GP) and Proctor. GP was an important malting barley up to 1990, and is highly susceptible to mildew. Proctor is an older variety, and has a moderate degree of partial resistance (Jones and Davies, 1985; Knudsen *et al.*, 1986). Both varieties have been used extensively in breeding programmes, and neither is known to carry any effective race-specific resistance gene. Samples of mildew were taken from each plot, from leaves with low densities of infection, at 3-week intervals, on 24 May, 14 June and 5 July. After the isolates had been purified so that each was a single clone of *E. graminis* f. sp. *hordei*, they were inoculated onto four detached leaf sections each of GP and Proctor by the method of Brown and Wolfe (1990), and the number of colonies formed were counted 7 days later. The test was carried out on 2 days, with 12 isolates sampled from each plot on each date being tested on one day and 12 more isolates on the second day.

If there had been adaptation of *E. graminis* f. sp. *hordei*, such that each variety had selected clones which were preferentially adapted to itself, we would have expected the ratio of the number of colonies (*C*) formed on Proctor to the number formed on GP to be higher for isolates sampled from Proctor than for those sampled from GP, and vice versa. This would express itself as a significant interaction between source variety and test variety in an analysis of variance (anova). In fact, no such interaction was seen (anova of log₁₀(*C* + 1): $F = 1.0 \times 10^{-4}$; 1 × 2003 d.f.; $P = 0.99$), and the ratios of colony numbers on Proctor to those on GP were very similar for isolates sampled from the two varieties (Table 7.1). Furthermore, there was no interaction with the date of sampling ($F = 0.24$; 2 × 2003 d.f.; $P = 0.8$), which indicates no significant evidence for progressive adaptation during the 6 weeks of the trial.

There was a significant interaction between isolates and test varieties ($F = 1.49$; 286 × 1722 d.f.; $P = 2 \times 10^{-6}$), which superficially indicates that isolates, regardless of their source variety, differ in adaptation to the two varieties. However, each isolate was only tested once, in a single inoculation. In other, similar tests, using a smaller number of isolates sampled in 1994, significant variety × isolate interactions were also observed, but interactions were not consistent between replicate experiments (R.E. Hague and J.K.M. Brown, unpublished). The relevance of the significant isolate × test variety interaction must therefore be doubted.

Table 7.1. Ratios of the number of colonies on detached leaves of Golden Promise (GP) to the number on Proctor, formed by isolates of *Erysiphe graminis* f. sp. *hordei* (barley powdery mildew), sampled from field trial plots of GP or Proctor at 3-week intervals in 1995.

Date	Source variety	
	GP	Proctor
24 May	2.70	2.82
14 June	3.39	3.26
5 July	3.64	3.18
Overall	3.12	3.08

There are several reasons why adaptation may not have been observed in this experiment, including:

- Variation in infection efficiency may not be expressed under the controlled conditions used in these experiments.
- The infection efficiency on detached leaves may not be related to that on living plants.
- Variation in fitness may be expressed in some way other than infection efficiency, such as latent period or sporulation.
- There may not have been enough time for selection to have acted on variation in fitness.
- The design of the field trials and the sampling scheme may not have been appropriate for the detection of fitness variation (see below).
- There may in fact have been no genetic variation in the *E. graminis* f. sp. *hordei* population for relative fitness on these two varieties.

However, there is a small amount of evidence from other experiments, mostly in the form of infection efficiencies on detached leaves, which does indicate the possibility of varietal adaptation in *E. graminis* f. sp. *hordei*. Wolfe *et al.* (1983), reviewing race survey data, observed a tendency for the number of colonies on a variety to be higher for isolates sampled from that variety than for isolates sampled from other varieties with the same resistance gene; their Table 7 illustrates this for varieties with *Mla1a*, *Mla12* and *Mla12 + Mlg*. Furthermore, Chin and Wolfe (1984, Table 6a) found that isolates sampled from plots of either Hassan (*Mla12*) or Wing (*Mla7 + Mlk1*), and virulent on both varieties, had a higher infection efficiency on their source variety than on the other variety. Newton (1989), testing ten isolates on GP and three partially resistant barley varieties, found a significant variety $\times$ isolate interaction, owing to a relatively high number of colonies formed by one isolate on one of the resistant varieties. Finally, three different barley variety mixtures, each consisting of three varieties with the same identified resistance genes, all had substantially lower mean levels of mildew infection than pure stands of the

same varieties (Wolfe *et al.*, 1981); this reduction may have been due to the action of unidentified, background resistance genes. Although acquisition of the appropriate race-specific virulence appears to be the key step in adaptation to a variety, a minor role for adaptation to the genetic background cannot be excluded.

Perhaps it would be more realistic for selection experiments to be conducted on field trial plots. For such trials to be appropriate, they must be designed in a way that allows the full extent of variation in the *E. graminis* population to be sampled. For instance, if *E. graminis* formed large foci of infection, as yellow rust does (Colwell, 1956), samples from even relatively large plots, like those used in the experiments described above (100 m²), would be unduly influenced by stochastic, spatial variation in clone frequencies. However, recent experiments on the establishment of epidemics by *E. graminis* f. sp. *hordei* have indicated that this need not be a serious concern. We have shown that epidemics are established by many clones, so that there is high genetic diversity within a field. The consequently large number of initial foci of infection means that no single focus is especially important in determining clone frequencies in the field as a whole, while the foci overlap considerably. The possible existence of localized, stochastic variation in clone frequencies therefore does not invalidate simple designs based on sampling from transects or from random points, provided that samples are taken from points more than 1 m apart. Finally, once an epidemic is established, migration between fields is slow – almost negligible – compared with the rate of epidemic development within fields; we can therefore treat experimental plots isolated by a reasonable distance (say 15 m) as independent experimental units (O'Hara, 1996).

A consequence of these results relates to the model of evolution of *E. graminis* f. sp. *hordei* populations of Hovmøller *et al.* (1993; also see Hovmøller *et al.*, Chapter 10 this volume). This model assumes that a mildew epidemic in a field is established by immigration of a large population of spores from nearby fields and that subsequent migration between fields is negligible. Our data largely support these assumptions.

Laboratory experiments on competition between mildew isolates have indicated that the process of selection may be much more complicated than has been supposed hitherto. A colour polymorphism (pink or white) in *E. graminis* f. sp. *tritici* allows highly efficient selection experiments to be designed, by mixing spores of two isolates, one pink, the other white, co-inoculating them onto detached leaves of a susceptible wheat variety and examining the isolates' relative infection efficiencies by counting colonies of the two colours. Experiments of this kind showed that the relative fitness of *E. graminis* f. sp. *tritici* isolates is density-dependent over a range of densities similar to that found in infected crops. In the most detailed series of tests, one isolate was fitter than the other at low densities, while the situation was reversed at intermediate or high densities. These results suggest that isolates of *E. graminis* f. sp. *hordei* differ in competitive ability (whether for space or for nutrients is not known), but the

absence of frequency-dependent selection indicates that colonies compete as strongly with other colonies of the same clone as with colonies of a different clone (O'Hara and Brown, 1996). The existence of density-dependent selection has serious consequences for experiments on pathogen fitness, since doubt must be cast on the validity of the results of any field trial in which samples are collected without regard for the density of mildew at the site of sampling, and of any laboratory experiment in which the density of inoculation is not controlled.

Durable and Non-Durable Resistance

The process of adaptation of *E. graminis* f. sp. *hordei* to barley varieties is now understood in outline, but important parts of that process have yet to be tested rigorously, while many details remain to be filled in. Some of the currently outstanding questions and challenges have been described above.

A major goal of plant breeding is durable disease resistance. Although there is no single genetic or phenotypic model for durable resistance (Johnson, 1984, 1993), certain features of cereal mildews can be identified as making the use of gene-for-gene resistance to control this disease a particularly non-durable strategy. These include rapid, long-distance dispersal of the pathogen, the prolific production of spores and the sexual phase of the life cycle, which allows combined resistance genes to be overcome. The first two aspects of mildew biology can be exploited in disease control by the use of variety mixtures (Wolfe and Barrett, 1980; discussed further by Brown, 1995b).

However, a crucial aspect of cereal mildew resistance which has contributed to non-durability is the mechanism of the gene-for-gene resistance itself. Evidence is emerging that this operates by specific recognition of a pathogen molecule by a host molecule (Staskawicz *et al.*, 1995), inducing host defence responses such as hypersensitive cell death. Any variation in DNA sequence which alters the structure of the pathogen's avirulence gene product may lead to that product not being recognized by the host's resistance gene product and so not inducing the host's response (Joosten *et al.*, 1994; Rohe *et al.*, 1995). It is sometimes claimed that all plant disease resistances will eventually be found to conform to the gene-for-gene mechanism, but this view is naïve. It is quite possible to imagine signals for the host defence response which do not involve specific, molecular interactions, including physical effects such as pressure by germ tubes or appressoria on the plant cell surface or penetration of the cell wall, or physiological effects such as a loss of turgor following infection of host cell. Since these are inevitable stages in the process of infection by powdery mildew fungi, it is reasonable to suggest that resistances which are triggered in ways such as these are likely to be more durable than gene-for-gene resistances.

The mechanisms of partial resistance (Carver, 1986) and *mlo* resistance (Jørgensen, 1992) differ from those of gene-for-gene resistance as it is presently understood (Görg *et al.*, 1993; Boyd *et al.*, 1995). The nature of the signals for partial resistance and *mlo* resistance are unknown; in the case of *mlo*, they do not appear to involve specific, molecular, host-pathogen recognition. Both of these types of resistance have been much more durable than gene-for-gene resistances. Variation in adaptation to partial resistance appears to be limited (Table 7.1 in this chapter; Wolfe *et al.*, 1981, 1983; Chin and Wolfe, 1984; Newton, 1989), while there has been little or no adaptation to *mlo* resistance (Andersen and Jørgensen, 1992; Lyngkjær *et al.*, 1995).

Data on cereal mildews suggest that race-specific, gene-for-gene resistances are generally more vulnerable to adaptation by the pathogen than are other types of resistance. Indeed, durable resistance could be defined in a negative sense, in that resistances which function by specific recognition of a particular pathogen molecule, usually inducing the hypersensitive response, are likely to be less durable than those which operate by different mechanisms. Part of the value of studying the gene-for-gene relationship is that knowledge about this system will enable pathologists to identify resistances which do not conform to the gene-for-gene model and are therefore likely to be more valuable in plant breeding.

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Virulence Dynamics and Genetics of Cereal Rust Populations in North America

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Introduction

The rust fungi historically and currently have been among the most important pathogens of wheat (*Triticum aestivum* L.) and oats (*Avena sativa* L.) on a world-wide basis. Cereal rust diseases have also been crucial in the conceptual development of host-parasite genetics. Biffen (1905) working with resistance in wheat to stripe rust caused by *Puccinia striiformis tritici* Westend was the first to show that disease resistance in plants was conditioned by Mendelian factors. Newton *et al.* (1930) were the first to demonstrate Mendelian inheritance of virulence in a plant pathogen with hybrid cultures of *P. graminis* on wheat. These early critical pieces of research undoubtedly influenced Flor (1971) in the conception and development of the gene-for-gene theory. Using *P. graminis tritici* as an example, Rowell *et al.* (1963) proposed using pairs of host lines and pathogen isolates that differ by only a single gene for resistance and virulence respectively, in examining gene-for-gene relations at the physiological and molecular levels.

Gene-for-gene relationships have been demonstrated in the wheat stem rust (*Puccinia graminis* Pers. f. sp. *tritici* Eriks. and Henn.) (Green, 1964), wheat leaf rust (*Puccinia recondita* Roberge ex Desmaz. f. sp. *tritici* Eriks. and Henn) (Samborski and Dyck, 1968, 1976), and oat crown rust (*Puccinia coronata* Cda.) (Nof and Dinooor, 1981) disease systems. These cereal rust diseases are particularly well suited for studying gene-for-gene relations at a population level since large scale surveys describing frequencies and distribution of physiological races of these fungi have been conducted both in Canada and the United States of America. Moreover, virulence frequencies to specific host resistance

genes can be directly estimated since single-gene, or near-isogenic host lines are now used as differentials.

The development of cereal cultivars with high levels of durable resistance to these three cereal rusts is a major goal of wheat and oat breeding programmes in North America. However, the results of these efforts have been mixed. Oat cultivars commonly lose effective resistance to crown rust after only a few years of cultivation; hard red spring wheats in North America are generally resistant to leaf rust and highly resistant to stem rust, while many of the winter wheats are susceptible to leaf rust. These discrepancies in effectiveness and longevity of host resistance are largely caused by differences in how the rust populations have responded to the introduction of host resistance genes. In this chapter, various attributes of cereal rust populations – racial diversity, virulence and molecular associations, and epidemiological factors – will be examined and compared with the ultimate goal of assessing how these influence the effectiveness and stability of host resistance.

Distribution and Diversity of Cereal Rust Populations

Survey results of *P. graminis tritici* in Canada in recent years have indicated that the same predominant races are found in the eastern province of Ontario and the western provinces of Manitoba and Saskatchewan (Harder *et al.*, 1994). This has been the case since the early years of the survey; Newton and Johnson (1946), and Green (1971) also found that races which were predominant over a period of years in western Canada were also found in other regions of the country. The number of different stem rust races in Manitoba and Saskatchewan has been low in recent years. From 1986 to 1994, an average of 6.2 races was found from an average of 310 single-uredinial isolates tested on 16 wheat differential lines. Diversity of the *P. graminis tritici* populations in Canada during this time was measured using the Shannon index (Groth and Roelfs, 1982, 1987b): $H_w = -\sum p_i \ln(p_i)$, where p_i = frequency of the i th phenotype. This index is indicative of the number of distinct phenotypes (races) and evenness of phenotype frequency distribution (Groth and Roelfs, 1987a). Between 1987 and 1994, the Shannon indexes have been relatively low, usually being very close to 1.0 (Fig. 8.1). As an example of the current low racial diversity, in the 1993 stem rust collections from wheat in Manitoba, 61% were race TPM (*Pgt* nomenclature [Roelfs and Martens, 1988]), 17% QFC, and 14% QCC (Harder *et al.*, 1994). These were also the predominant races of *P. graminis tritici* collected in the USA in 1992 (Roelfs *et al.*, 1993).

The present day *P. graminis tritici* population in the Great Plains region of North America reproduces strictly by the asexual propagation of urediniospores on wheat, barley and other compatible grass hosts. The asexual stem rust population overwinters on winter wheats in the southern Great Plains of the USA, and migrates every year on the southerly winds in the spring and

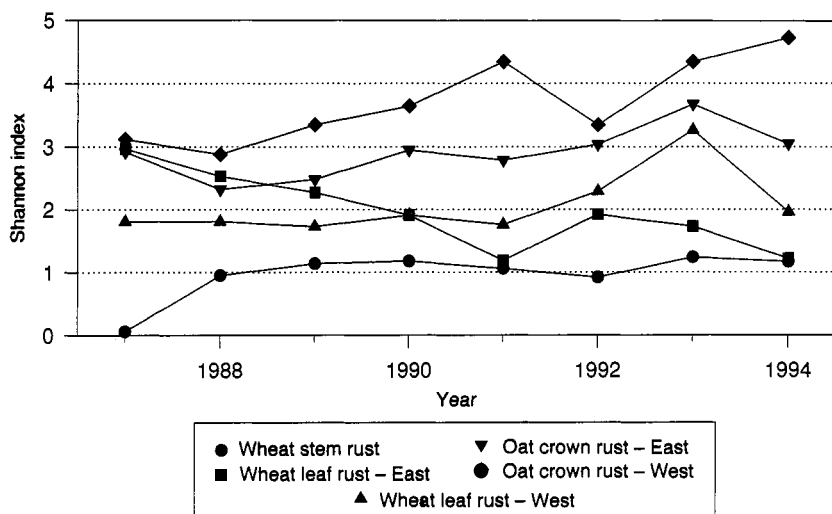


Fig. 8.1. Shannon indexes of phenotypic diversity (races) for *Puccinia graminis tritici* (wheat stem rust), *P. recondita tritici* (wheat leaf rust) and *P. coronata* (oat crown rust) in Canada from 1987 to 1994.

summer to the northern USA and Canada. However, the stem rust fungus had an important sexual component in its life cycle previous to the eradication of the alternate host common barberry (*Berberis vulgaris* L.), throughout most of North America in the 1920s. Groth and Roelfs (1987b) showed that the number of stem rust races detected in the US surveys declined from 30 in 1918, previous to removal of the alternate host, to only four in 1978. Shannon indexes declined from greater than 3.0 to 1.0 during this period. As summarized by Groth and Roelfs (1987b), removal of the alternate host has clearly contributed to the current low level of diversity, with only two or three predominant stem rust races throughout North America. An isolated sexual population of *P. graminis tritici* exists in the Pacific North-West of the USA, where barberry plants can still be found. One hundred races were detected from 426 isolates in the sexual population in 1975, compared with only 17 races from 2377 isolates from the asexual population (Roelfs and Groth, 1980).

The high level of host resistance found in many winter and spring wheat cultivars has reduced the effective population size of *P. graminis tritici* in North America, therefore also influencing racial diversity. Since the 1960s (Green, 1971, 1975) an increasing proportion of stem rust resistant winter wheats have been grown in the southern plains of the USA. The stem rust resistance genes *Sr6*, *Sr24*, and *Sr31* in the US winter wheats condition effective resistance to the current predominant races TPM and QCC. Resistance in the winter wheats greatly reduces the size of the overwintering stem rust population. Race TPM is virulent to stem rust resistance derived from the wheat

cultivar Triumph. Use of the Triumph resistance in the southern Great Plains (Roelfs and Groth, 1980) may have selected race TPM. Most of the hard red spring wheats grown in the northern USA and Canada have stem rust resistance derived from Thatcher (Kolmer *et al.*, 1991), which is conditioned by two recessive genes that have not been given *Sr* designations. The hard red spring wheats with Thatcher background combined with *Sr7a*, *Sr9b*, *Sr11*, *Sr12* and *Sr16* (Dyck, 1993) are highly resistant to stem rust. Stem rust uredinia cannot be found in farm fields planted to these wheats. The cultivation of highly resistant winter and spring wheats may have effectively 'bottle-necked' *P. graminis tritici* by allowing only a few races to reproduce and be maintained in the population.

In North America the wheat leaf rust fungus, *P. recondita tritici* reproduces only by the clonal propagation of urediniospores. North American species of the alternate host *Thalictrum* are resistant to basidiospore infection. As is the case for stem rust, leaf rust infections overwinter in winter wheats grown in the southern USA, and the urediniospores are wind-blown into the northern USA and Canada. However, *P. recondita tritici* has greater overwintering ability compared with the stem rust fungus. Overwintering infections of leaf rust can be found at more northerly latitudes (Chester, 1946; Roelfs, 1989). This has allowed regional populations of leaf rust races to develop (Kolmer, 1992a). In the 1995 leaf rust survey in Canada, only four of 35 races could be found in both the eastern population of Ontario and Quebec, and the western population of Manitoba and Saskatchewan (J. Kolmer, 1995 unpublished results). Leonard *et al.* (1992) also attributed regional leaf rust populations in the USA to areas where overwintering occurs.

The *P. recondita tritici* population in Manitoba and Saskatchewan has had a higher level of racial diversity in recent years (Fig. 8.1), and historically (Kolmer, 1991b) compared with wheat stem rust. From 1987 to 1994, an average of 18.2 races tested on 16 near-isogenic lines were detected from an average of 210 single-uredinial isolates. The winter wheats grown in Texas, Oklahoma and Kansas, where the leaf rust population overwinters are susceptible (Marshall, 1988), or often have only a single effective gene for leaf rust resistance when released. Cultivars with single seedling resistance genes lose effective resistance within a few years owing to the selection of virulent races. Use of different single resistance genes in different cultivars has resulted in a number of races being selected and maintained in the leaf rust population.

The *P. recondita tritici* population in eastern Canada originates from a combination of rust that overwinters on susceptible soft white winter wheats, and rust that has migrated from other regions of the USA or Canada. Isolates collected from the winter wheats in mid-late June, are races seldom if ever found in western Canada (Kolmer, 1992a). These collections are usually dominated by one or two races, which have most likely overwintered on the susceptible winter wheat. Collections from spring wheats in August have consisted of races found only in eastern Canada and races that are also found in western

Canada. These latter races most likely migrated from other regions of North America. Kolmer (1991a) noted a parallel change in frequencies of selected races in the eastern and western Canada populations of *P. recondita tritici*. Diversity in the eastern population (Fig. 8.1) has been lower compared with the western Canada population as most of the collections after 1990 have been made from winter wheats.

Puccinia coronata populations in Canada are extremely diverse in comparison with both wheat stem and leaf rust (Fig. 8.1). From 1987 to 1994 there was an average of 85 races, from an average of 149 isolates, from western Canada, and an average of 34 races from 108 isolates in eastern Canada. The sheer number of races suggests that sexual recombination is occurring. The alternate host of crown rust, buckthorn (*Rhamnus cathartica* L.), is commonly found with pycnial infections and aeciospores in Ontario (Fleischmann, 1967; Kolmer and Chong, 1993). The aeciospores are usually virulent to oats and/or rye. Fleischmann (1967) and Chong (J.Y. Chong, Winnipeg, 1996, personal communication) have isolated the same crown rust races from buckthorn and oats in eastern Canada. The crown rust population in Ontario is highly localized, cycling between the local buckthorn and oats. Virulence survey data has suggested that in Ontario little migration from other crown rust populations in North America occurs (J.Y. Chong, Winnipeg, 1996, personal communication).

In Manitoba and Saskatchewan, local sexual populations of crown rust may also originate from locally infected buckthorn plants. However, urediniospores probably also migrate from sexual populations in Minnesota, where infected buckthorn plants are common. There is some evidence that a limited amount of crown rust migrates from oats grown along the Texas Gulf coast to the northern USA and Canada (K.J. Leonard, St. Paul, 1996 personal communication). A combination of local and long distance inoculum from sexual and asexual origins contributes to the high levels of racial diversity currently observed in *P. coronata* populations in western Canada.

The crown rust populations in Ontario and Manitoba are distinct. In 1990, only seven races were found in both populations (Chong and Kolmer, 1993). The two populations differ in frequencies of virulence to resistance genes that have never been used in oat cultivars. This difference is not recent, as Fleischmann *et al.* (1963) also noted differences between the two populations. The virulence differences in the two populations are most likely due to the different sources of inoculum for each population.

Genetic associations in cereal rust populations

Virulence survey data of *P. graminis tritici*, *P. recondita tritici* and *P. coronata* populations in North America have been examined for population structure based on the distribution of virulences among isolates. If virulences are

randomly distributed, then racial diversity should be relatively higher in sexual populations compared with populations in which asexual reproduction maintains non-random associations between virulences. Groth and Roelfs (1987a) have examined how virulence associations and differing virulence frequencies affect the Shannon index of diversity. Associations between virulence and molecular markers can be used to determine genetic relatedness of rust races within a population.

Roelfs and Groth (1980) assessed the effect of sexual versus asexual reproduction on *P. graminis tritici* by comparing the distribution of virulences to specific *Sr* genes in these two populations in North America. The sexual population had a Shannon index of 1.78, compared to 0.53 for the asexual population (Groth and Roelfs, 1982). The lower diversity in the asexual population could be attributed to the clustering, or non-random distribution of virulences that was found in this population. Almost all isolates in the asexual population had virulence to either 6 to 7 or 9 to 11 stem rust resistance genes. In the sexual population, numbers of virulences per isolate were symmetrically distributed from 2 to 10. The distribution of virulence differences between pairs of isolates was also bimodal in the asexual population, with most isolate pairs differing by either 1 to 2, or 7 to 9 virulences. In the sexual population, the virulence differences between isolates were nearly randomly distributed. Alexander *et al.* (1984) examined the degree of association between pairs of virulences in the two stem rust populations by using contingency tables and the *G* statistic (Sokal and Rohlf, 1981). In the asexual population, 62 of 65 virulence pairs were non-randomly associated with either positive or negative association. In the sexual population, 24 of 46 pairs were non-randomly associated. The associations between virulences were more frequent and stronger in the asexual population compared with the sexual population.

Races in the asexual *P. graminis tritici* population were grouped into six clusters (Roelfs and Groth, 1980). Races within each cluster were closely related for virulence to host differential lines, while between clusters, races were highly dissimilar for virulences. These distantly related race groups have been found in the wheat stem rust population over the course of the virulence surveys (Green, 1971, 1975). Non-random virulence associations in the asexual wheat stem rust population have been relatively stable over time, even though the predominant races have changed (Alexander *et al.*, 1984). Roelfs (A.P. Roelfs, St. Paul, 1996, personal communication) has hypothesized that the current race clusters are representative of *P. graminis tritici* genotypes that were present in North America at the time of barberry eradication, or that the race clusters represent different asexual populations that were introduced from Europe.

In cereal rust fungi, isozyme and DNA-based molecular markers can provide additional insight into population structure since these markers by themselves are presumably unaffected by host selection. Rust isolates may differ greatly for virulence, yet have identical molecular phenotypes, indicating

that they may have diverged by host selection from a common ancestral genotype. Burdon and Roelfs (1985b) examined the relationship between isozyme and virulence variation in the asexual North American *P. graminis tritici* population. They found that grouping isolates by isozyme genotypes also grouped races that were closely related for virulence. The maximum number of virulence differences between isolates with the same isozyme genotype was 3.0, with an average of 1.6. The average virulence difference between isolates in different isozyme groups was 10.9. The isozyme markers grouped the isolates into six clusters which corresponded almost exactly with clustering using virulence markers. Isozyme variation was found among isolates only in one race cluster. The near complete association between isozyme genotypes and races has been maintained by asexual reproduction. In contrast, isozyme genotypes and races were not associated in the sexual *P. graminis tritici* population (Burdon and Roelfs, 1985a).

P. recondita tritici populations in Canada have also been examined for virulence associations. Characteristic non-random associations between pairs of virulences in the eastern and western wheat leaf populations as determined with contingency tables and the G statistic are given in Tables 8.1 and 8.2. A

Table 8.1. Virulence associations to pairs of leaf rust resistance genes in wheat in the eastern (Ontario, Quebec) population of *Puccinia recondita* f. sp. *tritici* in Canada in 1990 and 1995 as measured by the G statistic.

Virulence pair	1990	1995
<i>Lr2a, Lr2c</i>	NS ^b	+ ^c
<i>Lr2a, Lr3ka</i>	— ^d	—
<i>Lr2a, LrB</i>	—	—
<i>Lr2a, Lr14a</i>	+	+
<i>Lr2c, Lr3ka</i>	+	NS
<i>Lr2c, Lr11</i>	—	—
<i>Lr2c, LrB</i>	+	+
<i>Lr2c, Lr14a</i>	—	—
<i>Lr24, Lr3ka</i>	—	—
<i>Lr24, LrB</i>	—	—
<i>Lr24, Lr14a</i>	+	+
<i>Lr3ka, Lr11</i>	—	—
<i>Lr3ka, LrB</i>	+	+
<i>Lr3ka, Lr14a</i>	—	—
<i>Lr11, LrB</i>	—	—
<i>Lr11, Lr14a</i>	+	+
<i>LrB, Lr14a</i>	—	—

^aContingency table test (Sokal and Rohlf, 1981).

^bNon-significant association ($P > 0.05$).

^cSignificant negative association ($P < 0.05$).

^dSignificant positive association ($P < 0.05$).

non-random distribution of virulences would be expected since the wheat leaf rust populations in Canada reproduce asexually. Non-random virulence associations in wheat leaf rust populations have arisen by genetic linkage, host selection or random chance. Virulences to resistance genes *Lr3ka* and *Lr30* are genetically linked, within four map units (Samborski and Dyck, 1976; Kolmer, 1992b). Isolates that are virulent to *Lr30* are almost always virulent to *Lr3ka*. The two loci may be so closely linked that a single mutation affects both. The same allele at one locus in *P. recondita tritici* conditions avirulence to resistance alleles *Lr2a* and *Lr2c* (Dyck and Samborski, 1974). An independent allele in *P. recondita* differentially inhibits the expression of avirulence to *Lr2a* and *Lr2c*. Isolates heterozygous for both the avirulence and inhibitor alleles are avirulent to *Lr2a*, and virulent to *Lr2c*. Isolates avirulent to *Lr2a* and virulent to *Lr2c*, have been common races in eastern Canada since the start of the virulence survey (Kolmer, 1991a). Races in the western population are all either virulent to both *Lr2a* and *Lr2c*, or avirulent to both genes. This virulence association is

Table 8.2. Virulence associations to pairs of leaf rust resistance genes in wheat in the western (Manitoba and Saskatchewan) population of *Puccinia recondita* f. sp. *tritici* in Canada from 1987 to 1995 as measured by the G^2 statistic.

Virulence pair	1987	1988	1989	1990	1991	1992	1993	1994	1995
<i>Lr1, Lr2a</i>	— ^b	—	—	—	—	—	—	NS ^c	* ^d
<i>Lr1, Lr24</i>	+ ^e	+	+	+	+	+	+	NS	NS
<i>Lr1, Lr26</i>	*	*	+	+	+	+	+	NS	NS
<i>Lr1, Lr3ka</i>	*	*	*	*	*	*	+	+	*
<i>Lr1, Lr11</i>	*	*	—	—	—	—	NS	NS	*
<i>Lr1, Lr30</i>	*	*	*	*	*	*	+	+	*
<i>Lr2a, Lr24</i>	—	—	—	—	—	—	NS	+	+
<i>Lr2a, Lr26</i>	*	—	—	—	—	—	—	NS	NS
<i>Lr2a, Lr3ka</i>	*	*	*	*	*	*	—	—	—
<i>Lr2a, Lr11</i>	*	+	+	+	+	+	+	NS	NS
<i>Lr2a, Lr30</i>	*	*	*	*	*	*	—	—	—
<i>Lr24, Lr26</i>	*	+	+	+	+	+	+	+	+
<i>Lr24, Lr3ka</i>	*	*	*	*	*	*	NS	—	—
<i>Lr24, Lr11</i>	*	*	—	—	—	—	—	—	—
<i>Lr26, Lr3ka</i>	*	*	*	*	*	*	+	NS	NS
<i>Lr26, Lr11</i>	*	—	—	—	—	—	—	—	—
<i>Lr26, Lr30</i>	*	*	*	*	*	*	+	NS	NS
<i>Lr3ka, Lr11</i>	*	*	*	*	*	*	NS	NS	+
<i>Lr3ka, Lr30</i>	*	*	*	*	*	*	+	+	+

^aContingency table test (Sokal and Rohlf, 1981).

^bSignificant negative association ($P < 0.05$).

^cNon-significant association ($P > 0.05$).

^dExpected cell(s) in 2×2 contingency table < 5 , G test not conducted.

^eSignificant positive association ($P < 0.05$).

the most characteristic difference between races in the eastern and western populations. Isolates that are virulent to *Lr2a* and avirulent to *Lr2c* have never been found in survey collections, or in genetic studies with *P. recondita tritici*.

Host selection can also generate non-random virulence associations. In the western population virulences to *Lr24* and *Lr26* have been positively associated (Table 8.2) because winter wheat cultivars with both resistance genes have selected races with virulences to the two genes. Also in the western population virulences to *Lr1* and *Lr2a* have been dissociated since 1975 (Table 8.2) because these genes have been present in different cultivars (Kolmer, 1989a). In eastern Canada isolates that are virulent to *Lr2c*, *LrB* and *Lr3ka*, and avirulent to *Lr2a* and *Lr14a*, have been the common leaf rust races for 35 years (Kolmer, 1989b). Only two of the virulence associations listed in Table 8.1 changed between 1990 and 1995, reflecting the relative racial stability of the leaf rust population in eastern Canada.

Kolmer *et al.* (1995) examined the relationship between virulence and molecular polymorphism in *P. recondita tritici* with representative isolates from eastern and western Canada. Cluster analysis based on virulence phenotypes and randomly amplified polymorphic DNA (RAPD) markers separated the isolates into two major groups. Isolates avirulent to *Lr2a* and virulent to *Lr2c* and commonly found in the eastern population comprised one group, and isolates virulent or avirulent to both alleles and found mostly in the western population comprised the second group. Virulences to 19 differential near-isogenic lines distinguished 37 races among the 64 isolates, while only 15 RAPD phenotypes could be distinguished using ten random DNA primers. The RAPD markers were more effective in distinguishing between the two major groups of isolates; however the virulence markers were much more effective in distinguishing between isolates within the clusters. Isolates within the clusters had similar RAPD phenotypes, yet could have very different virulence phenotypes. There was only limited molecular variation compared with the abundant virulence variation.

Kolmer and Chong (1993) examined the distribution of virulences in the eastern and western *P. coronata* populations in Canada. The number of virulences per isolate, and number of virulence differences per isolate pair, closely approximated a random distribution for both populations. Since the virulences were nearly randomly distributed, few associations between pairs of virulences could be found in either population. An average of only 1.23 and 3.94 non-random virulence associations to ten *Pc* genes from 1974 to 1990 were found in the eastern and western populations, respectively. Non-random associations between pairs of virulences did not persist for more than 3 years in either population. The near-random distribution of virulences, and the lack of persistent virulence associations, indicate that sexual recombination must occur annually in oat crown rust populations in eastern and western Canada.

Increase of Virulences Selected by Host Resistance Genes

The long-term effectiveness of rust resistance genes in cereal crops is dependent on the rate at which races with virulence to host resistance increase and become prevalent in the rust population. Genetic diversity and population structure will influence the speed in which new virulences are selected and incorporated into rust populations as a whole.

Changes in *P. graminis tritici* races in Canada, from the start of the surveys in 1919 to the mid-1960s, were characterized by a succession of races that originated from the different asexual race clusters (Table 8.3). Race HFL was a prevalent race from 1919 to 1933 (Newton and Johnson, 1946). This race was virulent to commonly grown cultivars such as Marquis and Red Fife. Race MCC became the most important stem rust race in Canada in 1934 because of virulence to Ceres wheat (Green, 1975). Race MCC differed from HFL by eight virulences. Ceres wheat was replaced in popularity by cultivars that had stem rust resistance derived from Hope and H-44 (*Sr2*, *Sr7b*, *Sr9d*, *Sr17*), and by Thatcher (Kolmer *et al.*, 1991). Race TMR predominated from 1950 to 1954 since it was virulent to the Hope, H-44 and Thatcher resistance. This highly damaging race differed from MCC by ten virulences. Selkirk wheat, with *Sr6* which conditioned resistant to race TMR, was commonly grown from 1955 to 1965.

Table 8.3. Progression of prevalent *Puccinia graminis tritici* (wheat stem rust) races in Canada. Races are identified with the *Pgt* three letter nomenclature (Roelfs and Martens, 1988) or the Canadian race number (Green, 1981) designation in parentheses. Numbers of virulence differences between races are in square brackets. Virulence formulae indicate single-gene wheat stem rust differentials for which the isolates are virulent.

Years	Prevalent races	Virulence formula
1919–1933	HFL (C1)	7b, 8a, 9d, 9g, 14, 15, 21, 36
	↓ [8]	
1934–1949	MCC (C17)	5, 7a, 7b, 9g, 10, 14, 15, 17
	↓ [10]	
1950–1956	TMR (C10)	5, 7b, 9a, 9b, 9d, 9e, 9g, 10, 11, 13, 14, 14, 21, 36
	↓ [10]	
1957–1963	MCC (C17)	5, 7a, 7b, 9g, 10, 14, 15, 17
	↓ [7]	
1964–1968	TML (C18)	5, 7a, 7b, 9d, 9e, 9g, 10, 11, 14, 21, 36
	↓ [1]	
1969–1974	TPL (C33)	5, 7a, 7b, 8a, 9d, 9e, 9g, 10, 11, 14, 21, 36
	↓ [1]	
1975–1993	TPM (C53)	5, 7a, 7b, 8a, 9d, 9e, 9g, 10, 11, 14, 17, 21, 36
	↓ [7]	
1990–1995	QCC	5, 9d, 9g, 10, 13, 14, 15, 17, 21

The changes in the *P. graminis* population after 1954 have been unrelated to the resistance genes used in the spring wheats. After the release of Selkirk, race TMR declined, and MCC became prevalent again. Starting with Manitou in 1966, cultivars with the Thatcher stem rust resistance and additional specific *Sr* genes have been released and grown in western Canada. The Thatcher type cultivars have been highly resistant to stem rust. Race TML, which differed from MCC by seven virulences, became the most prevalent race from 1964 to 1968, and was in turn replaced by TPL in 1969, and TPM in 1975 (Table 8.3). Races TML, TPL and TPM are highly related, differing only in virulence to *Sr8* and *Sr17*. This line of stem rust races may have become established because of virulence to the Triumph stem rust resistance in the US winter wheats.

In 1990 race QCC became common in the stem rust population in Canada. This race is highly avirulent to the spring wheat and many of the winter wheat cultivars; however it has virulence to resistance gene *Rpg1* in cultivated barley. In 1993 QCC was the most prevalent stem rust race collected from barley in Manitoba and Saskatchewan, while TPM was the most commonly collected race from wheat (Harder *et al.*, 1994). Races QCC and TPM differ in virulence to at least seven stem rust differential lines (Fox *et al.*, 1995), and also have different ribosomal DNA banding patterns. The large number of virulence differences, and the different molecular backgrounds make it unlikely that QCC originated as a mutant from a stem rust race cluster in the asexual Great Plains population. This new race may have originated in the *P. graminis tritici* sexual population of the Pacific North-West and was subsequently introduced into the Great Plains population.

Changes in *P. recondita tritici* races in western Canada can be explained almost entirely by the introduction of cultivars with single resistance genes followed by selection of virulent races. In the initial years of the leaf rust survey from 1931 to 1944, the eastern and western populations had the same predominant races. Race 9 was commonly found in both populations (Kolmer, 1991a). This period was before the widespread introduction of leaf rust resistant wheat cultivars in North America. Spring wheat cultivars with *Lr14a* were introduced in 1937, and winter wheats with *Lr3* were released in 1943 (Kolmer, 1991a). Race 9 declined rapidly because of avirulence to *Lr3* and *Lr14a* and was replaced by races 2 and 5, which had virulence to both these genes and differed from race 9 by five and four virulences, respectively (Kolmer, 1991a). An isolate of race 9 had virulences and RAPD markers that widely separated it from the current two major clusters of *P. recondita tritici* isolates in Canada (Kolmer *et al.*, 1995). Isolates of race 9 may have comprised an additional major cluster of *P. recondita tritici* races before cultivars with *Lr3* and *Lr14a* were released. This race has not been collected from cultivated wheat for over 20 years in western Canada (Kolmer *et al.*, 1995).

After the decline of race 9, leaf rust races in western Canada have changed by a stepwise addition of virulences, with all races being derived from one

original race cluster (Kolmer *et al.*, 1995). The cultivars Lee (*Lr10*) and Selkirk (*Lr10*, *Lr14a*, *Lr16*), were released in 1950 and 1955, respectively. Virulence to *Lr10* and *Lr16* was highly associated with race 2, which increased to nearly 100% of the western population from 1968 to 1978 (Kolmer 1989b, 1991a). Race 2 started to decline when spring wheats in the USA with *Lr1* and *Lr2a* were released in the early 1970s and races with virulences to these genes began to increase in 1976 (Kolmer, 1989b). US winter wheat cultivars with genes *Lr11*, *Lr24* and *Lr26* have been grown since 1987 and leaf rust races with virulences to one or more of these genes increased (Fig. 8.2). In 1993 virulence to *Lr3ka* began to increase rapidly because of winter wheat cultivars with *Lr3ka*. These selected virulences were initially limited to the races in which they were originally found. Lack of sexual recombination prevented the initial spread of selected virulences into many different races in the population. From 1987 to 1992, non-random associations between virulences to *Lr1*, *Lr2a*, *Lr11*, *Lr24* and *Lr26* remained constant (Table 8.2). Virulence to *Lr11* arose in a race that was avirulent to *Lr1*, *Lr24* and *Lr26*, and virulent to *Lr2a*. Virulence to *Lr24* and *Lr26* arose in a race that was avirulent to *Lr2a* and virulent to *Lr1*. Virulence to *Lr3ka* has increased in races that are avirulent to *Lr2a* and virulent to *Lr1*.

As frequencies of the selected virulences increased, they also became more evenly distributed among different races in the population. In 1988 virulence to *Lr11* was 11%, and was found in only four races; however, by 1993 virulence to *Lr11* was at 60%, and was found in 13 races. Associations between pairs of virulences also changed as virulences became more evenly

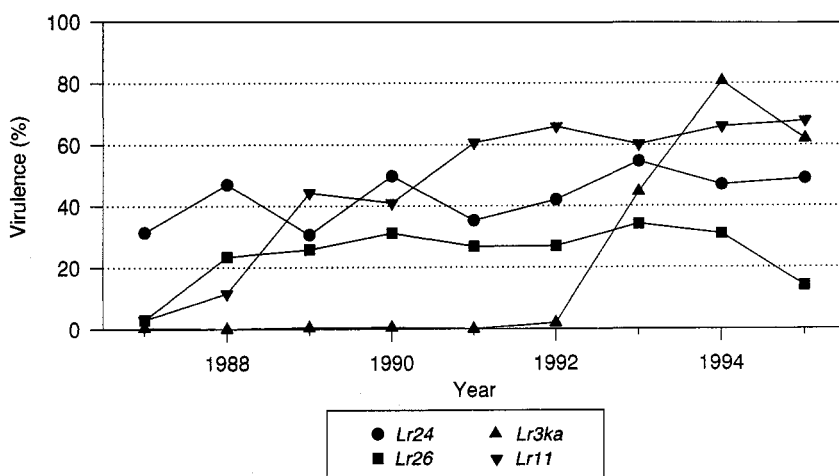


Fig. 8.2. Frequency (%) of *Puccinia recondita tritici* (wheat leaf rust) isolates with virulence to resistance genes *Lr3ka*, *Lr11*, *Lr24* and *Lr26* in western Canada from 1987 to 1995.

distributed among races. In 1993 non-random associations between *Lr1* and *Lr24*, *Lr1* and *Lr26*, *Lr1* and *Lr11*, *Lr2a* and *Lr24*, *Lr2a* and *Lr26*, became non-significant (Table 8.2). Negative association between *Lr2a* and *Lr24* changed to positive in 1994 and 1995; non-random associations between *Lr2a* and *Lr26*, and *Lr2a* and *Lr11*, changed to non-significant in 1994 and 1995. Although the virulences in *P. recondita tritici* are effectively linked owing to asexual reproduction, recurrent mutation and selection of virulences over time will distribute virulences among different race phenotypes.

Heterozygosity for virulence genes is a very important source of genetic variation that is not readily apparent in the virulence surveys. Since rust fungi are dikaryotic, different single-uredinial isolates may have identical combinations of avirulence and virulence on differential lines, yet may be genetically distinct if the isolates are heterozygous or homozygous at different virulence loci. Individual *P. recondita tritici* isolates have been shown to be heterozygous for virulence alleles at a number of loci (Samborski and Dyck, 1976). Kolmer (1992b) determined at a population level that many *P. recondita tritici* isolates from Manitoba and Saskatchewan were heterozygous for virulence to resistance genes *Lr3ka*, *Lr11*, *Lr17* and *Lr30*, even though the number of isolates with virulence to these genes was low. Virulences to *Lr11* and *Lr3ka* were at 12 and 0.0%, respectively, in the western Canada leaf rust population in 1988 (Fig. 8.2). By developing a random mating population of isolates collected in the 1988 western Canada survey, Kolmer (1992b) estimated that virulence allele frequencies to *Lr11* and *Lr3ka* were 45 and 47%, respectively. Almost all of the leaf rust isolates that were avirulent to these genes in the survey would have been heterozygous for virulence. The abundant pre-existing heterozygosity helps to explain why virulence to *Lr11* and *Lr3ka* increased so rapidly in western Canada after 1988 and 1992, respectively. In contrast little, if any, virulence heterozygosity was detected to *Lr24* and *Lr26*. Isolates in the survey that were avirulent to these genes, were almost all homozygous for avirulence. The lack of heterozygosity in the *P. recondita tritici* population to *Lr24* and *Lr26* may explain why virulences to these genes have not increased as rapidly, or to levels as high as virulences to *Lr11* and *Lr3ka* (Fig. 8.2).

Resistance in oats to *P. coronata* has been short-lived because of the rapid distribution of selected virulences into the rust population. This undoubtedly results from regular sexual recombination in both the eastern and western populations of this pathogen. In 1983 the oat cultivar Woodstock with *Pc39* was released in Ontario and in 1985 virulence to *Pc39* was found in one crown rust isolate in Ontario. By 1990, however, virulence to *Pc39* had increased to 77% (Fig. 8.3) and occurred in 36 races (Chong and Kolmer, 1993). Oat cultivars with *Pc38* and *Pc39* have been grown in Manitoba since 1984. In 1988 virulence to *Pc38* and *Pc39* were 1 and 0%, respectively. However, by 1990 virulence to *Pc38* and *Pc39* was 59 and 44%, respectively (Fig. 8.3), and virulence to both genes was found in 39 races (Chong and Kolmer, 1993).

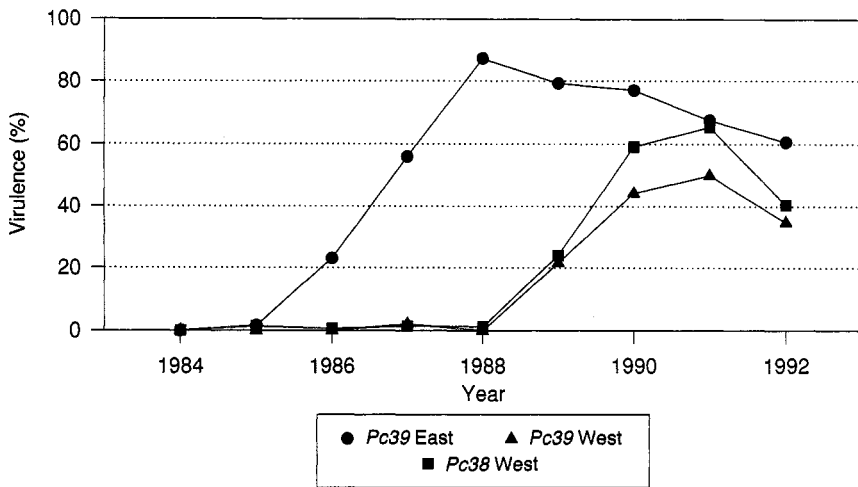


Fig. 8.3. Frequency (%) of *Puccinia coronata* (oat crown rust) isolates with virulence to resistance gene *Pc39* in eastern Canada and *Pc38* and *Pc39* in western Canada from 1984 to 1992.

Conclusions

The three rust populations are distinct in all population characteristics that have been examined. The presence or absence of sexual reproduction and effective population size are probably the most important factors that influence the racial diversity, population structure, and host selection of virulences in cereal rust populations (Table 8.4).

The *P. graminis tritici* and *P. coronata* populations in North America represent two extremes. The Great Plains wheat stem rust population has very low racial diversity, no geographic subdivisions, and a non-random distribution of genetic markers that has resulted in clusters of distantly related genotypes. In contrast, oat crown rust populations are highly diverse, with different race populations in eastern and western Canada, and virulences that are randomly distributed within both populations. Virulent races of oat crown rust are selected by newly introduced host resistance genes, while in the last 40 years the introduction of spring wheat cultivars with different resistance genes has had no selective effect on the wheat stem rust population. The abundance of sexual reproduction in *P. coronata* and the totally asexual nature of *P. graminis tritici* is obviously the most important reason why these two cereal rusts differ so greatly at a population level.

However, *P. recondita tritici* in North America is also asexual and has basic epidemiological characteristics in common with wheat stem rust; yet leaf rust populations are considerably more racially diverse, have different regional race populations and respond quickly to the selective effects of host resistance.

Table 8.4. Population attributes of *Puccinia graminis tritici* (wheat stem rust), *Puccinia recondita tritici* (wheat leaf rust) and *Puccinia coronata* (oat crown rust) in North America.

Population attributes	<i>P. graminis</i>		<i>P. recondita</i>		<i>P. coronata</i>	
	Great Plains	Pacific North-West	East	West	East	West
Racial diversity	Low	Medium	Medium	Medium	High	High
Geographic subpopulations	No	—	Yes	Yes	Yes	Yes
Sexual(S)/asexual(A) reproduction	A	S	A	A	S	S
Non-random genetic association	High	Low–medium	High	Medium–high	Low	Low
Effective host resistance	High	—	^a Low/ ^b variable	^c Low/ ^d high	Low	Low

^aWinter wheats in Ontario and Quebec are leaf rust susceptible.

^bSoft red winter wheats in the USA vary for leaf rust resistance.

^cWinter wheats in the southern US plains are often leaf rust susceptible.

^dSpring wheats in the northern USA and Canada are leaf rust resistant.

These differences can probably be explained by differing levels of host resistance in wheat to leaf and stem rust, which directly influences the effective size of the two rust populations. The stem rust resistance in many winter wheats, combined with the popularity of early maturing cultivars, has restricted the urediniospore population of *P. graminis tritici* in the southern plains of the USA. The highly resistant spring wheats further reduce population size. In contrast, many winter wheat cultivars do not have effective leaf rust resistance, and in years which favour leaf rust epidemics, even resistant winter and spring wheats can have moderate levels of leaf rust severity (Marshall, 1988; Kolmer *et al.*, 1991). The ability of leaf rust to overwinter in areas further north than stem rust, combined with the lower levels of leaf rust resistance in wheat, has resulted in a larger effective population size compared with stem rust. In a larger population, individual isolates with mutations to virulences that confer a selective advantage would have a better chance of surviving and increasing in frequency.

Durable resistance to cereal rusts can be obtained only by employing resistance genes that maintain effective levels of resistance even in the face of highly variable and dynamic pathogen populations. It is fortunate that spring wheats in North America have had high levels of stem and leaf rust resistance for over 30 years. The Thatcher-derived stem rust resistance has been highly effective, and the stem rust population has not changed in response. The

adult-plant leaf rust resistance genes *Lr13* and *Lr34* by themselves, and in combination with seedling resistance genes, have also maintained effective resistance, even though the *P. recondita tritici* population changes rapidly in response to the seedling resistance genes used in the winter wheats. Durable leaf rust resistance in winter wheats and crown rust resistance in oats will remain difficult, if not impossible to achieve if cultivars with only one or two seedling resistance genes continue to be released. Alternative approaches such as adult-plant resistance or complex combinations of resistances must be tried if there is to be any hope of obtaining long-lasting resistance to these diseases.

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Interpreting Population Genetic Data with the Help of Genetic Linkage Maps

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The population genetics of plant pathogens is increasingly being investigated with the use of molecular markers such as restriction fragment length polymorphisms (RFLPs), randomly amplified polymorphic DNA (RAPDs) (Welsh and McClelland, 1990; Williams *et al.*, 1990), amplified fragment length polymorphisms (AFLPs) (Zabeau and Vos, 1993) and derived, polymerase chain reaction (PCR) markers (SCARs; Paran and Michelmore, 1993; McDermott *et al.*, 1994). Molecular markers are more abundant than the formerly used biochemical and phenotypic characters such as isozymes, anastomosis groups and morphological traits. Furthermore, in contrast to virulence and fungicide resistance, they are assumed to be selectively neutral (Michelmore and Hulbert, 1987). This statement is based on the fact that only a small percentage of the eukaryotic genome contains functional genes, so that the chances of finding polymorphic DNA markers in non-coding regions are much higher. However, if neutral loci are linked to loci under selection, their neutral behaviour depends on the amount of recombination in the organism.

Based on observations from the barley powdery mildew pathogen, the haploid, obligate biotrophic fungus *Erysiphe graminis* f. sp. *hordei*, we demonstrate here the effects of linkage to loci under selection and of a mixed reproductive system on putatively neutral DNA markers in finite pathogen populations. We stress the importance of genetic linkage information for the interpretation of population genetic data. The use of markers linked to traits under selection to answer specific population genetic questions is demonstrated.

The Barley-*Erysiphe graminis* System

The biology and general population genetic aspects of the barley powdery mildew pathogen are treated in this volume (Brown *et al.*, Chapter 7 and Hovmøller *et al.*, Chapter 10 this volume) and elsewhere (Jørgensen, 1988; Wolfe and McDermott, 1994). Two aspects of the biology are of direct relevance for this chapter: the gene-for-gene interaction between host and pathogen, and the mixed reproductive system which allows for both sexual and asexual transmission of fungal spores from one growing season to the next.

Numerous qualitative resistance genes in the host are matched by corresponding avirulence genes in the pathogen (Jørgensen, 1994). This has been exploited extensively in resistance breeding. However, newly introduced resistances are usually overcome by matching virulence alleles within a few years (Brown *et al.*, 1991, 1993).

E. graminis hordei overwinters mainly in the asexual (conidial) state on the autumn-sown crop. Epidemics develop during the growing season and may lead to a build-up of airborne conidial inoculum. At the end of the conidial mass-propagation phase in June, fusion of fertilization hyphae of opposite mating type leads to the formation of drought-resistant cleistothecia containing the asci with ascospores. Fungal populations survive the summer months without a barley crop both asexually on volunteer plants and as cleistothecia on straw. The emerging winter crop is then inoculated by a mixture of sexually and asexually maintained fungal individuals.

Populations, Markers and Maps

Airborne samples of *E. graminis hordei* were collected across Europe using a Schwarzbach mobile spore trap mounted on a car roof (Schwarzbach, 1979; Limpert, 1987). Field isolates were collected from infected leaves and transferred twice to detached leaf segments of a susceptible variety to obtain pure isolates. Ascospores were collected from cleistothecia germinated over susceptible detached leaf segments (Haemmerli *et al.*, 1994). Virulence tests were carried out and scored as described (Wolfe and McDermott, 1994) using Pallas near-isogenic lines (Kølster *et al.*, 1986). RAPDs and SCARs were generated from lyophilized conidia with the methods described by McDermott *et al.* (1994) (Fig. 9.1). Genetic linkage of virulence and molecular markers was determined in a cross between two isolates from France and Czechoslovakia, respectively. The mating type of 42 progeny isolates was determined by back-crossing them to both parents. Avirulence loci, molecular marker loci and the MAT locus were mapped (Haemmerli *et al.*, 1994) using the program MAP-MAKER (Lander *et al.*, 1987), Version 3.0, with multipoint linkage and the Kosambi mapping function. Map regions of special interest were saturated

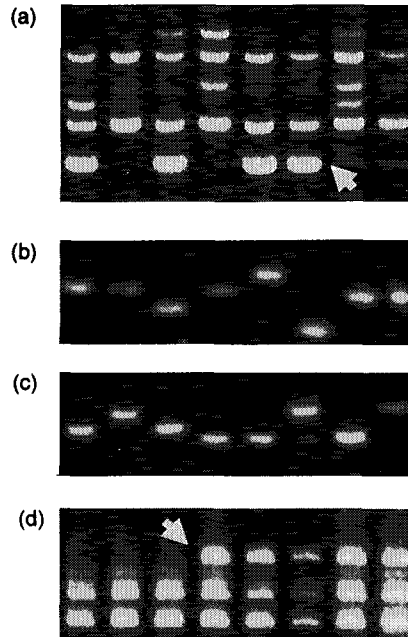


Fig. 9.1. Molecular markers in *E. graminis* f. sp. *hordei*. (a) Amplifications of eight random isolates from a field population in Switzerland with Primer PJ-02. The arrow indicates the band designated PJ-02-1020. (b–d) Amplifications of eight random European isolates with SCAR primer pairs SPEGH-07A (b), SPEGH-M18 (c), SPEGH-V02 (d). The arrow in (d) indicates the band scored as marker.

further with markers using bulked segregant analysis (Michelmore *et al.*, 1991). Genetic linkage information for the molecular markers discussed here is given in Table 9.1.

Linked Virulence Alleles Remain Associated

The need for optimal exploitation of resistance genes has stimulated attempts to model the interaction of *E. graminis hordei* populations with the host at local and regional levels (Hovmøller and Østergård, 1991; Østergård and Hovmøller, 1991; Hovmøller *et al.*, 1993). Hovmøller *et al.* (1992) pointed out that positive gametic disequilibrium of virulence alleles occurs through selection when the two corresponding resistance genes coexist in a region or variety. Negative disequilibria would be selected only if there is a cost of virulence. Brown (1995) developed these ideas further and showed that the frequency of unselected alleles can also increase temporarily as a result of hitch-hiking selection if there is initial disequilibrium and a low frequency of sexual reproduction.

Table 9.1. Recombination distances of genetic markers in *E. graminis* f. sp. *hordei*.

MAT group		Va7 group		Va13 group		M18 group
MAT		PJ-02-1020		A/Va13		SPEGH-M18
	3.3 cM		7.0 cM		6.9 cM	
SPEGH-V02		A/Va7		SPEGH-E07A		
	0.7 cM					
SPEGH-Q17						
	0.2 cM					
SPEGH-Q12						
	0.4 cM					
SPEGH-M16						
	0.2 cM					
SPEGH-U12						

Distances were calculated using the Kosambi mapping function on data of 160 F₁ individuals. The distance between MAT and the molecular marker is based on 46 F₁ individuals.

We found evidence for both negative and positive associations of virulence alleles in our European population surveys between 1989 and 1991 (Wolfe and McDermott, 1994). While there was no constant trend for most virulence pairs across years and regions, the virulence alleles Va9 and Vk were in positive disequilibrium in all sampled populations, significantly so in most. Haemmerli (unpublished results) found that Va9/Vk was the only combination of 24 virulence alleles tested which deviated significantly from random-mating expectations in all of the samples taken in Switzerland from each of two fields in May (conidial population), June (cleistothecia) and September (from volunteers). Neither virulence has been selected for recently in Switzerland.

Our findings can be readily interpreted with the help of a mildew genetic map: the avirulence loci *A/Va9* and *A/Vk* have been mapped to the same linkage group (Jørgensen, 1988; Haemmerli *et al.*, 1994) separated by approximately 25 cM. This represents the closest linkage among the virulence loci investigated in our survey. Positive association of virulence alleles was pronounced for these linked loci, probably because genetic linkage reduces the effect of sexual recombination as the force breaking up such associations. Thus, recombination must occur in regional and local populations of *E. graminis hordei* at levels that prevent stable gametic disequilibrium among unlinked loci. Loose linkage, however, seems to be sufficient to favour the associating force of combined selection over the dissociating force of recombination.

Neutral Molecular Markers May Behave Non-Neutrally

The advent of PCR-based molecular markers has spawned many studies of the distribution of genetic variation in pathogen populations. Allele frequencies at

neutral loci are often used to estimate gene flow among putatively isolated populations (Boeger *et al.*, 1993; McDermott and McDonald, 1993). This information may then be used, for example, to optimize the use of resistance genes in different regions. However, some authors have pointed out that the concepts developed for ideal natural populations should be used cautiously with pathogen populations (Milgroom and Lipari, 1995). In this section, we demonstrate that in organisms like *E. graminis hordei* with no obligate sexual stage and mass asexual propagation, selection can also affect neutral loci. The following paragraphs describe our stepwise progress so as to underline the fact that conclusions from allele frequency data should not be made unless complete linkage information is available.

In our European pathogen collection from May and June 1990, the RAPD band PJ-02-1020 (Fig. 9.1a) indicated strong subdivision among 34 population samples expressed by a G_{ST} value of 0.36. This corresponded to the amount of subdivision that we had observed for some virulence loci matching recently introduced resistance genes, e.g. *Mla13*. While subdivision at virulence loci can be readily explained by the distribution of host resistance genes, subdivision at loci which are not selected would normally be explained as resulting from limited gene flow (about 0.3 immigrants per generation and population in our case). Alternatively, the locus PJ-02-1020 could be associated with a gene which is exposed to differential selection across the continent.

When we plotted virulence allele frequencies against molecular marker frequencies, it became obvious that marker PJ-02-1020 was not common in samples with a high frequency of Va13 (Fig. 9.2). In populations where the Va13 allele was present in more than 10% of the sample, we detected significant negative disequilibrium between the virulence and the presence of the molecular marker. This suggested association between the two loci. However, what appeared to be linkage between the RAPD locus and the *A/Va13* locus turned out to be an example of hitch-hiking (Wolfe and Knott, 1982), once we had produced the genetic map (see Table 9.1): locus PJ-02-1020 is relatively closely linked to the *A/Va7* locus in the *E. graminis hordei* genome, whereas *A/Va13* belongs to another linkage group. Therefore, the correlation between the absence of marker PJ-02-1020 and the presence of Va13 is not a result of linkage. More likely, the RAPD marker was rare in the source population where selection for Va13 originally took place. Va7 and Va13 were often selected simultaneously, which led to predominating genotypes containing Va13 and Va7 but not the molecular marker. This was expressed by high frequencies of Va7 in the samples with a high proportion of Va13 (see connected data points in Fig. 9.2).

The observed subdivision for the 'neutral' RAPD locus is therefore most probably caused by selection at a linked avirulence locus. In species with prominent clonal propagation, DNA markers cannot be regarded simply as being neutral unless their linkage to loci under selection is fully understood. Even then, they may be affected by hitch-hiking selection. Population genetic

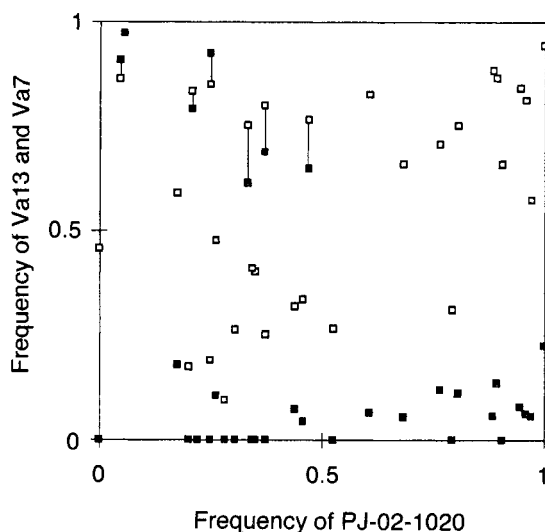


Fig. 9.2. Frequency of virulence alleles Va13 (■) and Va7 (□) plotted against frequency of molecular marker PJ-02-1020 in 34 European populations of *E.graminis* f. sp. *hordei*.

concepts such as that of gene flow should therefore be applied only if the data indicate no association between neutral loci and loci under selection.

The Spread of a New Virulence: a Genetic Sweep of Multiallelic Loci?

With the exception of *mlo*, all resistance genes currently used against barley powdery mildew are matched by corresponding avirulence genes in at least parts of the pathogen population. Virulence alleles can arise by deleterious mutations in a functional avirulence gene, or they can be the result of a favourable mutation in a non-functional avirulence gene. While both possibilities are conceivable, avirulence genes cloned in several host–pathogen systems have as yet all been of the former type (Keen, Chapter 20 and Dangl, Chapter 21 this volume). As the probability for loss of function through mutation is much higher than for gain of function, it is possible that phenotypically identical virulence alleles arise frequently. It is then a question of selection whether these mutants are maintained or lost. Once established, frequent recombination leads to the integration of virulence alleles into the genetic background of the population in which they are selected. Alternatively, if large areas are occupied by the resistance gene causing strong selection for the virulence allele, the virulent population fraction may consist of a limited number of genotypes. Virulent genotypes migrate eventually over large distances and

initiate new epidemics on previously resistant hosts, as occurred recently with the *Mla13*/*Va13* gene-for-gene pair. We were able to show that the predominant genotype responsible for the breakdown of the resistance in Switzerland was identical to a common genotype in Czechoslovakia, where *Mla13* has been used since the early 1980s (Wolfe and McDermott, 1994).

If the spread of a new virulence is rapid, genetic diversity in the virulent population fraction is reduced because of the limited time available to introduce the new allele into the original genetic background through recombination. Charlesworth (1992) coined the term 'genetic sweep' for such a reduction in genetic diversity. This effect is even more pronounced for loci in the genetic neighbourhood of the locus under selection because linkage limits recombination (Begun and Aquadro, 1992).

In order to determine if the spread of the *Va13* virulence in Europe was accompanied by such a genetic sweep, we analysed *E. graminis hordei* samples from seven European regions where *Mla13* barley had been grown. The isolates were tested for virulence against *Mla13*. Alleles at the locus *SPEGH-E07A* linked to *A/Va13* (6.9cM, four alleles detected in the sample) and the unlinked locus *SPEGH-M18* (five alleles) were determined by PCR (Fig. 9.1b and 9.1c). Nei's (1973) gene diversity, H , which represents the probability of sampling two isolates with different alleles, was assessed for virulent and avirulent fractions in all population samples. Based on the observed allele frequencies, samples of the original size were generated randomly by a computer and gene diversities calculated. This was repeated 5000 times for each sample to generate confidence limits for H . In order to allow for comparisons of the values for the two loci, H was normalized by its maximum value of $1 - 1/r$ at a locus with r alleles.

Table 9.2 shows the results of the gene diversity analysis. No significant difference in diversity could be detected between virulent and avirulent fractions at either locus. There was also no clear trend across the tested populations, indicating more diversity in one fraction. Our analysis of virulent and avirulent subpopulations did not show a reduction of allelic variation through selection for virulence and therefore provided no evidence for a genetic sweep.

Nei's gene diversity (H) can be divided into its components originating from different levels of subdivision, as demonstrated by Beckwitt and Chakraborty (1980). Table 9.3 shows how much of the total gene diversity at each locus was found within population fractions (virulent and avirulent on *Mla13*, respectively), among those fractions within regions and among regions in Europe. For both loci, similar patterns of diversity distribution were observed: roughly three-quarters of the observed diversity was found within virulent and avirulent subpopulation fractions, and about one-quarter was found among virulent and avirulent fractions. Only very little regional differentiation was found. In other words, allelic diversity in the *E. graminis hordei* population is evenly distributed among European regions but not among population fractions, which differ in their ability to overcome *Mla13*. This argues for a diverse

Table 9.2. Gene diversity in European barley mildew samples at loci unlinked (*SPEGH-M18*) and linked (*SPEGH-E07A*) to *Va13*.

	Collection ¹							Total
	A/CS	CH	CS/PL	DK	D-O	D-W	GB	
Sample size	47	26	71	62	36	22	10	274
Frequency of <i>Va13</i>	0.30	0.96	0.76	0.06	0.92	0.50	0.10	0.52
Gene diversity ²								
<i>SPEGH-M18</i> (avir)	0.80	0.00	0.37	0.88a	0.53	0.65	0.77	0.81a
<i>SPEGH-M18</i> (vir)	0.73	0.68	0.64	0.90ab	0.68	0.36	0.00	0.70ab
<i>SPEGH-E07A</i> (avir)	0.44	0.00	0.75	0.63b	0.00	0.37	0.80	0.61b
<i>SPEGH-E07A</i> (vir)	0.51	0.50	0.61	0.47b	0.44	0.58	0.00	0.65b

¹ Mildew samples were collected in May and June 1990 along the following routes:

A/CS: St.Pölten–Wien–Bratislava–Kúty

CH: Lausanne – Geneva

CS/PL: Hranice – Ostrava – Krapkowitz

DK: Flensburg – Kolding; Korsør – Roskilde – Vordingborg

D-O: Dresden – Hernsdorfer Kreuz

D-W: Meckenheim – Bingen – Ludwigshafen

GB: Leeds – Newark – Cambridge

² Different letters indicate values significantly different from each other with $P < 0.05$ determined by Monte Carlo tests with 5000 resamplings. No comparisons were made when the number of virulent or avirulent isolates was less than 3 (GB and CH).

Table 9.3. Hierarchical distribution of gene diversity at loci unlinked (*SPEGH-M18*) and linked (*SPEGH-E07A*) to *Va13* in a European sample of *Erysiphe graminis* f. sp. *hordei*.

Gene diversity <i>H</i>	Locus	
	SPEGH-M18	SPEGH-E07A
Within fractions virulent/avirulent on <i>Mla13</i> (%)	71	76
Among fractions virulent/avirulent on <i>Mla13</i> (%)	28	21
Among regions (%)	1	3

virulent founder population which spread from the areas where *Mla13* was originally used. With all attempts to explain the spread of virulence, one has to keep in mind that different regions may have different 'colonization' histories.

An Approach to Estimating the Frequency of Sexual Recombination Using Markers Around the Mating Type Locus

Haldane (1932) hypothesized that species with a sexual cycle followed by several steps of clonal reproduction would be evolutionarily the most

successful. Many plant pathogenic fungi, including *Erysiphe*, possess a mixed reproductive system as a key feature of their life cycle. The asexual stage, often in association with mass propagation, allows the maintenance of favourable combinations of alleles at genetically unlinked loci. The potential for recombination, on the other hand, creates new genotypic variants which, together with new mutations, account for adaptations to a changing environment. For *Erysiphe graminis*, there has only been one report of parasexuality (Menzies and MacNeill, 1986) but we could not confirm these results (Haemmerli, unpublished data). Sexual reproduction is most likely essential for genetic recombination. Its role of combining in a single genome formerly separated alleles, conferring virulence or pathogenicity, makes the frequency of sexual reproduction a topic of primary importance also for disease management. Brown and Wolfe (1990) used changes in linkage disequilibria among samples collected from the summer and the autumn population to estimate the proportion of isolates originating from ascospores in the autumn. Brown *et al.* (Chapter 7 this volume) discuss the reason why large sample sizes are required for such an approach.

We have attempted to estimate the frequency of sexual reproduction by concentrating on the mating type locus and its genetic neighbourhood. In a fully sexual population, the frequency of the two mating type alleles is restored to 0.5 after each round of sexual reproduction, no matter what their frequency was in the population that formed the cleistothecia. Six molecular markers resulted from our attempts to saturate the region around the mating type locus; their map locations are given in Table 9.1. As linkage to the mating type locus is based on only 42 F_1 progeny, the calculated distance is associated with a large variance and may in fact be much tighter: complete correlation between the *MAT* and *SPEGH-VO2* alleles was found in more than 90 isolates with known mating type from different laboratories in Europe (S. Christiansen, Roskilde; J.K.M. Brown, Norwich; V. Caffier, Grignon; and U.A. Haemmerli, Zürich, 1995, personal communications). We are currently producing mating type information for a larger set of progeny isolates.

The *MAT*-linked markers were tested on two ascospore populations collected from cleistothecia in two different barley fields. *SPEGH-VO2* alleles did not differ significantly from a 1:1 ratio, whereas all other markers were at frequencies different from 0.5 (Table 9.4). Considering that random drift operates every season at the population bottleneck in the late summer months, only markers in very tight linkage to the mating type locus will remain at 50% over years in ascospore populations. This has been the case for *SPEGH-VO2* in two different field samples.

In order to test how linkage to the mating type stabilizes allele frequencies over time, we developed a genetic drift model for a diallelic locus *B* linked to the mating type locus *A* in an isolated population. The model assumes that the frequency s of sexual reproduction is independent of the mating type

Table 9.4. Frequencies of DNA markers linked to the mating type locus in cleistothecia of *E.graminis* f. sp. *hordei* collected from two barley fields.

Marker	Field			
	Triton	<i>n</i>	Narcis	<i>n</i>
SPEGH-V02	0.53*	350	0.48*	350
SPEGH-Q17	0.09	350	0.17	350
SPEGH-Q12	0.31	96		
SPEGH-U12	0.79	96		
SPEGH-M16	0.92	96		

n: Number of individuals tested for marker.

*Frequencies are not different from 0.5 with $P < 0.05$ (Binomial distribution).

frequency. Figure 9.3 shows the mating scheme which was used to derive the recursion formulae for the frequency of each genotype in the next generation.

With

- r = recombination frequency between two loci *A* and *B*,
- s = fraction of population originating from ascospores,
- N = size of population in autumn,
- t = generation,
- d = random drift factor, depending on N and frequency,

we get the ascospore frequency of genotype *AB* in generation $t + 1$:

$$f_{AB_{t+1}(\text{sex})} = f_{AB_t} \times f_{ab_t} \times (1 - r) + f_{aB_t} \times f_{AB_t} + f_{Ab_t} \times f_{aB_t} \times r \tag{1}$$

As only the possible matings are taken into account, this value has to be corrected by their total frequency which is derived from Fig. 9.3 as

$$f(\text{matings}) = 2 \times f_{A_t} \times (1 - f_{A_t}) \tag{2}$$

with the frequency f_{A_t} of one mating type allele. Combining equations (1) and (2) with the frequency of asexual progeny and the random sampling factor d for both population fractions we obtain

$$\begin{aligned} f_{AB_{t+1}} = & s / (2 \times f_{A_t} \times (1 - f_{A_t})) \times (f_{AB_t} \times f_{ab_t} \times (1 - r) + f_{aB_t} \times f_{AB_t} \\ & + f_{Ab_t} \times f_{aB_t} \times r) \times d_{\text{sex}} + (1 - s) f_{Ab_t} \times d_{\text{asex}} \end{aligned}$$

The formulae for the three other genotypes are derived in the same way. Random drift is simulated by drawing (with replacement) N individuals from a population with the calculated genotype frequencies and size N .

Median time to fixation, the time (in sexual generations) after which half of the simulated populations become fixed for one of the alleles at locus *B*, can be used as a measure for the stability of the gene frequencies. This was calculated from the model with a critical population size of $N = 100$ for different values of the recombination frequency r and the sexual reproduction rate s . The initial

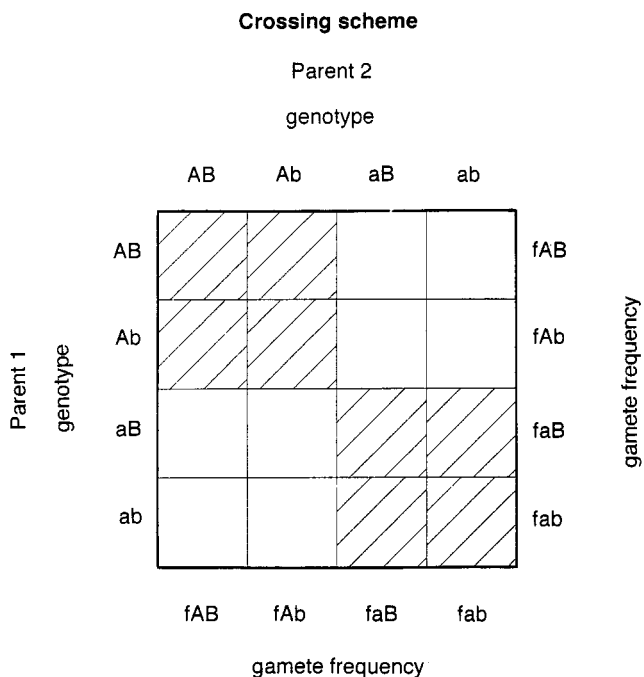


Fig. 9.3. Crossing scheme for two locus genotypes with alleles *A* and *a* at the mating type (*MAT*) locus. Hatched areas indicate mating incompatibility caused by identical alleles at the *MAT* locus.

frequency of 0.5 for genotypes *AB* and *ab* stands for maximum disequilibrium between the two loci (Fig. 9.4).

In the absence of sexual reproduction, fixation time is approximately N generations. With increasing sexual reproduction ratios, the linkage effect causes the fixation rates to increase up to a value of s between 2 and 3%. For higher rates of s , the effect of dissociation caused by sexual reproduction is stronger than the linkage effect and leads to a decrease in the fixation rate. Closely linked markers 'benefit' enormously from the proximity of the mating type locus (e.g. median fixation time for $r = 1$ cM is increased up to 16-fold compared with random drift). In practice, population size and sexual reproduction rate probably vary greatly across years and locations. Brändle *et al.* (1992) found more than 100 different *E. graminis hordei* genotypes in a field sample. N in founding populations in autumn may therefore largely exceed values of 100, which in turn means more stability of allele frequencies. N and s in the model have to be regarded as parameters representing theoretical average values of population size and sexual reproduction frequency.

The predictions made from our model may explain the surprisingly high number of polymorphisms that we found closely associated with mating type

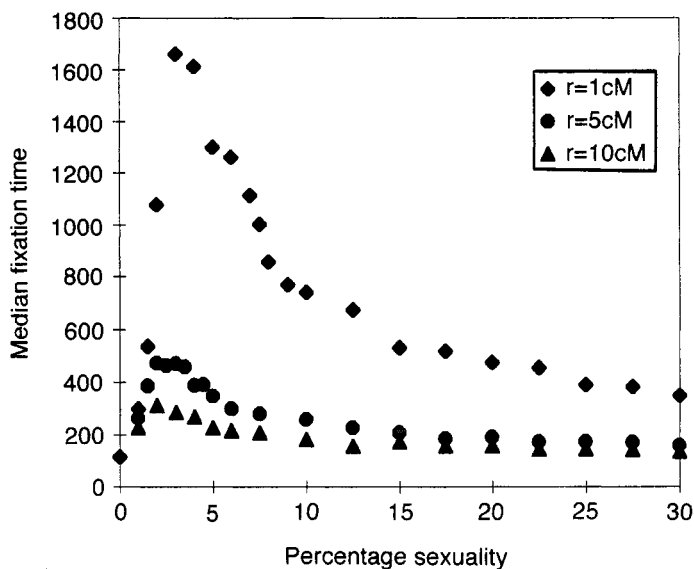


Fig. 9.4. Influence of sex rate s on the mean number of generations to fixation of alleles at a diallelic locus with recombination distances $r = 1$ cM, 5 cM and 10 cM from the mating type locus. 500 populations were simulated with a model assuming a critical population size of $N = 100$, constant sex rate until fixation and maximum initial disequilibrium.

by bulked segregant analysis (Haemmerli *et al.*, 1994). Assuming low sexual reproduction ratios, polymorphisms in close proximity to the *MAT* locus would remain in a population much longer than those at neutral or strongly selected loci.

Using the model, we generated frequency distributions of linked alleles at different distances from the *MAT* locus after 200 generations, with a population size of 100, r and s variable. These distributions were then compared with the frequencies of the marker SPEGH-V02 in 29 mildew populations collected across Europe in 1990 with the Mann-Whitney U -test for non-parametric variables. For the mating type locus itself ($r = 0$), the observed distribution fits the model for all sexual reproduction frequencies above 2%. For the calculated distance of 3.3 cM, sexual reproduction ratios between 1.5 and 15% would fit the expectations. It will be possible to make more precise estimates of the sexual reproduction frequency in *E. graminis hordei* with map distances for several markers based on larger F_1 populations.

Conclusions

Population genetic analysis of plant pathogens has long promised to result in cropping strategies which are ideally suited for the host-pathogen system

in question (McDonald *et al.*, 1989). McDermott and McDonald (1993) have suggested the application of classical population genetic theory to fungal populations. However, in organisms with mixed reproductive systems, prominent asexual stages can lead to hitch-hiking selection as we have shown for a putatively neutral DNA marker. Moreover, we have provided evidence that under conditions of limited recombination, combined selection for virulence may maintain virulence allele associations at very loosely linked loci. Our findings suggest that markers for population analysis ideally should be used only if their genetic linkage is known, and if they show no association with loci under selection.

Genetic linkage of molecular markers to selected loci may be exploited to answer specific population genetic questions. We have shown that multiallelic loci can be used to test hypotheses about reductions in genetic diversity following the emergence of new virulence. Sequence information of the virulence alleles themselves may in the future allow more precise descriptions of how virulent populations evolve. For the time being, haplotypes constructed from alleles at tightly linked loci could be substituted for alleles at the actual virulence locus.

Our second approach to a population genetic question using linkage map information addresses the frequency of sexual reproduction in *E. graminis hordei*. Only mating type alleles have an exactly predictable frequency after each round of sexual reproduction. We have outlined how this unique characteristic may be used to estimate the proportion of sexual reproduction in *E. graminis hordei* and in other organisms with a mixed reproductive system, such as human pathogens (Tibayrenc *et al.*, 1991).

The past few years have seen a mainly exploratory approach to population genetics in plant pathology. In a next step, specific hypotheses about pathogen populations may be tested. This will be made possible by precisely characterized molecular markers as well as by computer modelling.

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Modelling Virulence Dynamics of Airborne Plant Pathogens in Relation to Selection by Host Resistance in Agricultural Crops

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Introduction

In agricultural plant production systems, yield and quality of the crops have been much improved through breeding, for example by the introduction of genetically based disease resistance. In many areas, the agricultural systems are characterized by the presence of large areas of cultivated crops with identical or closely related host genotypes. Such systems are very different from natural ecosystems, where genetic variability in the host population is large, and the frequency of different host genotypes is a result of a balance between host, pathogens and environmental factors (Burdon, 1993).

In agricultural systems, selection by host resistance generally has a strong influence on pathogen population dynamics. For biotrophic plant pathogens, such as cereal mildews and rusts, where virulence genes in the pathogens are matched by host resistance genes, selection is likely to be the most powerful dynamic force relative to other forces such as mutation, migration and genetic drift (Østergård and Hovmøller, 1991).

Host induced selection results in increased frequencies of virulence genotypes with genes matching the resistance genes in the host crops. This has been demonstrated in a large number of virulence survey studies in cereal mildews and rusts (for reviews see proceedings edited by Jørgensen (1991) and Zeller

and Fischbeck (1992)). Further, many survey studies have demonstrated the existence of gametic disequilibria between virulence loci, i.e. non-random associations of alleles at different loci (Wolfe and Knott, 1982; Alexander *et al.*, 1984; Royer *et al.*, 1984; Welz, 1988; Brown and Wolfe, 1990; Hovmøller and Østergård, 1991a; Kolmer, 1992). Gametic disequilibria may arise from different types of selection, intermixture of populations with different gene frequencies, random genetic drift and mutation (Hedrick *et al.*, 1978; Wolfe and Knott, 1982; Østergård and Hovmøller, 1991).

The usefulness of results from virulence surveys and population genetic studies depend considerably on knowledge about the mechanisms and causes of genetic variation in pathogen populations. One successful methodology to improve insight into these mechanisms has been the development of mathematical models. The first simple genetically based models in plant pathology were used to estimate fitness values of single virulence genes on the basis of observed gene frequency dynamics over time (Leonard, 1969; Grant and Archer, 1983). Other models were developed to study virulence dynamics in multilines and variety mixtures with different resistance genes in the mixture components (Barrett, 1980; Østergård, 1983; Marshall, 1989). The models often assumed independence between different loci in the pathogen, and selection against virulence genes unnecessary for pathogen infection and growth. However, these assumptions may reduce the predictive value of the models because multilocus associations are common in pathogen populations, and until now there has been little experimental evidence for the existence of selection against unnecessary virulence genes (Parlevliet, 1981; Bronson and Ellingboe, 1986).

Recently, models have been developed for analysing survey data with multilocus associations among virulence loci, and taking into account selection defined by complex combinations of host resistance genes (Østergård and Hovmøller, 1991; Hovmøller *et al.*, 1993). These models were inspired by the population biology and genetics of *Erysiphe graminis* f. sp. *hordei*, the causal agent of powdery mildew on cultivated barley (*Hordeum vulgare*). This chapter reviews these models, with emphasis on analysis of a number of common themes which have been the subject of much debate in virulence surveys: (i) estimation of selection forces, (ii) gametic disequilibria between virulence genes, and (iii) dynamics of unnecessary virulence genes. Finally, the implications of the models for durability of host resistance genes, i.e. the time period in which the genes provide satisfactory disease control, are illustrated by simulations of the rate of change in virulence genotype and gene frequencies under different selection regimes.

Models of Genotype Frequency Dynamics

The models presented here take into account the most important aspects of the biology of *E. graminis*, which is a haploid and biotrophic fungal pathogen with

aerially dispersed spores, and with virulence genes being matched by resistance genes in the barley host (Moseman, 1959; Jørgensen, 1992). The models include two-locus models comprising the features of both sexual and asexual reproduction, and multilocus models taking only asexual reproduction into account. The influence of selection by host resistance genes was analysed in the case of no fitness costs of virulence genes that were unnecessary for pathogen infection of specific varieties. In the following, the biology of *E. graminis*, and the mechanisms of host induced selection and its consequences for population structure and dynamics are described in more detail.

Biology of the powdery mildew–barley system

E. graminis is prevalent in many barley growing areas, where it is found on the host as mycelia and colonies of asexually reproduced spores, and as cleistothecia containing ascospores being a product of sexual reproduction (Jørgensen, 1988; Wolfe and McDermott, 1994). The annual pathogen cycle starts in autumn, when the new autumn-sown host crops are infected by airborne spores (Fig. 10.1). The primary source of inoculum is likely to be local fields in which barley crops have been grown in the previous growth season, a minor fraction may be migrant spores from adjacent areas, and a very small proportion may even come from neighbouring regions/countries (see Hermansen *et al.*, 1978; Hovmøller, 1996).

When the population establishes on barley crops in autumn, the number of mildew colonies increases through several cycles of asexual reproduction of spores on the growing host (shaded area in Fig. 10.1). Usually, the population size (number of colonies) decreases again in the following winter because of unfavourable climatic conditions for mildew reproduction and winter damage of infected host tissue. In spring, spores produced on autumn-sown barley infect emerging crops of spring barley, and the population size increases rapidly on the growing hosts of both autumn- and spring-sown varieties (main epidemic phase). The population increases in size until host senescence gradually causes a decrease in the quality of host tissue as a substrate for the pathogen. At this stage, cleistothecia with asci may develop on host plants as a result of sexual reproduction, but according to a Danish investigation the ascospores are not released until autumn, i.e. in September, October and November (Smedegård-Petersen, 1967).

The population size is reduced to its minimum at crop harvest time. However, volunteer plants may emerge some few days after crop harvest, offering new suitable host tissue for the pathogen. The annual cycle is completed when asexually reproduced conidia from volunteer plants and green side tillers, and sexually reproduced ascospores are randomly dispersed onto new barley crops in autumn. The proportion of spores resulting from sexual reproduction varies between locations and years according to the number of cleistothecia

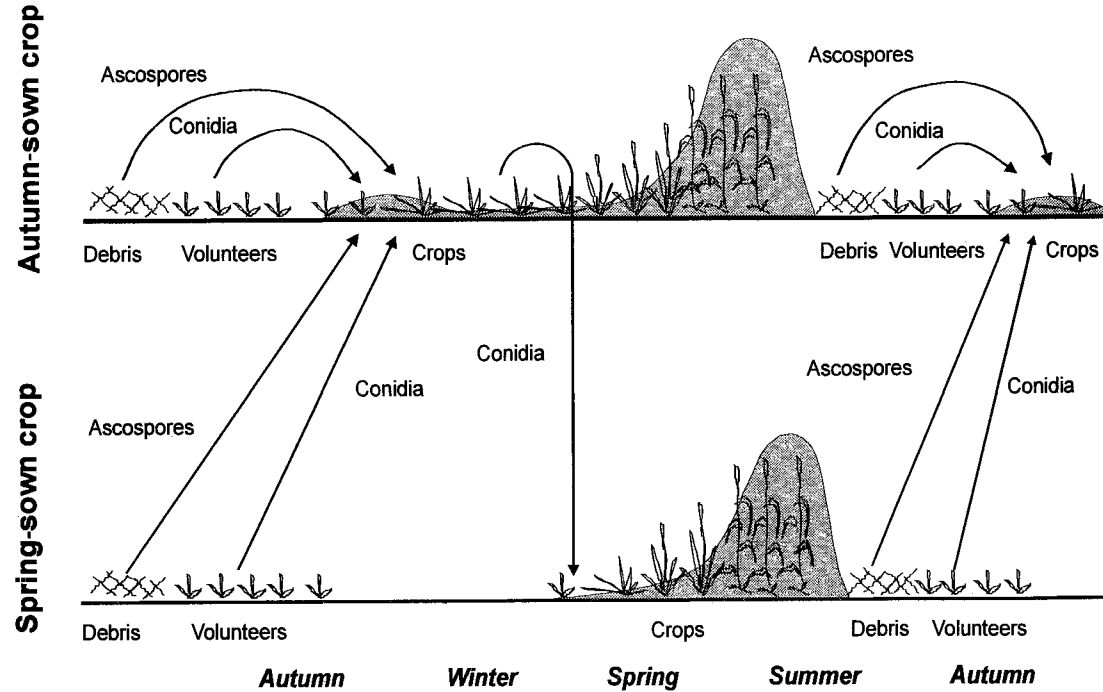


Fig. 10.1. Annual cycle of *Erysiphe graminis* f. sp. *hordei* in Europe. Shaded area represents population size measured by relative number of powdery mildew colonies on host plants, and arrows designate key points of spore dispersal.

developed in the previous growth season, the rate of release of ascospores, and in relation to weather conditions and amount of green host tissue favouring asexual reproduction.

The dispersal of airborne spores onto the newly emerged host varieties results in different mildew subpopulations growing on these varieties. The genetic differences between subpopulations are determined by the presence of host resistance genes, which induce selection such that only virulent genotypes are capable of reproducing on the varieties. The example in Fig. 10.2 illustrates a case with three resistance genes, *Mlx*, *Mly* and *Mlz*. Both *Mlx* and *Mly* are present in the variety grown in field I, *Mlz* is present in the variety in field II, and no resistance gene is present in the variety in field III. The corresponding virulence loci in the pathogen each have two alleles designated V_i and A_i (virulence and avirulence corresponding to resistance gene *Mli*, $i = x, y$ or z). Spores of the two genotypes possessing both V_x and V_y can infect the variety in field I, spores of the four genotypes possessing V_z can infect the variety in field II, and spores of all eight genotypes can infect the variety in field III.

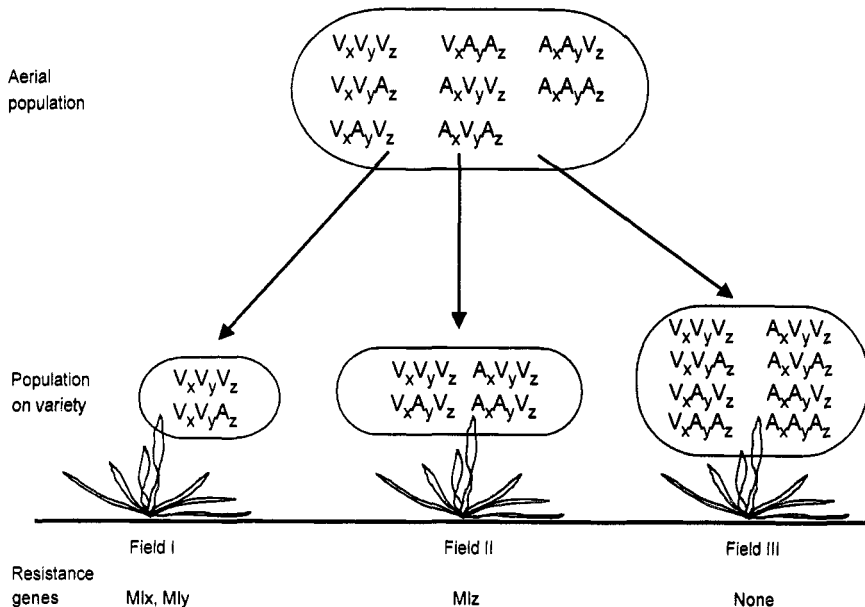


Fig. 10.2. Three-locus genotypes in the aerial powdery mildew population, and in the mildew subpopulations on three host varieties possessing different (or no) mildew resistance genes.

Prediction of changes in virulence genotype frequencies

The frequencies of virulence genotypes on specific host varieties can be calculated from the genotype frequencies in the aerial population infecting the varieties (Østergård and Hovmøller, 1991; Hovmøller *et al.*, 1993). Let f_i denote the frequency of spores of genotype i in the aerial population being dispersed in autumn ($i = 1, \dots, 2^n$ where n = number of virulence loci considered). Let f_{ij} denote the frequency of spores of genotype i establishing colonies on host variety j ($j = 1, \dots, m$ where m = number of host varieties considered). Further, let u_{ij} be the probability that spores of genotype i are established on host variety j , where $u_{ij} = 1$ or 0 depending on whether genotype i is capable of reproducing on variety j (virulent) or not (avirulent). Then the genotypic frequencies among colonies established on variety j equal

$$f_{ij} = f_i \times u_{ij} / w_j, \quad (1)$$

where the normalizing factor w_j is defined such that $\sum_i f_{ij} = 1$, i.e. w_j equals $\sum_i f_i \times u_{ij}$. This factor can be interpreted as the average fitness of aerial spores on variety j relative to the fitness of a virulent spore.

The aerial population at the end of the growing season is made up of spores produced by the mildew colonies present on the different host varieties. The genotypic frequencies in this population, f'_i , are weighted averages of the genotypic frequencies in the subpopulations. In cases where spores for new infections come almost entirely from within the field during the epidemic phase, and the relative distribution of green foliage (substrate for the pathogen) of different varieties is constant during the growth season, f'_i can be expressed as:

$$\begin{aligned} f'_i &= \sum_j f_{ij} \times w_j \times s_j / w' \\ &= f_i \times [\sum_j u_{ij} \times s_j] / w' \end{aligned} \quad (2)$$

where s_j is the relative area of variety j within the considered barley area, and $w' = \sum_j w_j \times s_j$. Eqn 2 assumes:

- asexual reproduction;
- no mutation or migration of spores to and from the considered area;
- dispersal of spores on emerging host varieties according to their relative area;
- identical spore production of different genotypes capable of infecting the same variety;
- identical spore production on different varieties of a genotype capable of infecting these varieties.

In Eqn 2 the probability of survival of successful infections on varieties equals 1. This probability can be included as a parameter, v_j (Hovmøller, 1993), i.e.

$$f'_i = f_i \times [\sum_j u_{ij} \times v_j \times s_j] / w'' \quad (3)$$

where, in its simplest form, $v_j = 1$ or 0 depending on whether an established infection survives or not (e.g. as a result of fungicide application), and w'' is a normalizing factor equal to $\sum_j w_j \times v_j \times s_j$.

When both autumn and spring sown varieties are grown in the same region, the genotypic frequencies in the aerial population at the end of the growing season can be calculated assuming further that:

- Number of spores per unit area infecting emerging autumn and spring sown varieties are of the same order of magnitude.
- Total number of spores produced on autumn and spring sown varieties are of the same order of magnitude.

Defining S as the relative area in summer of autumn sown crops, and s_j as the relative area of each specific variety in summer, the genotypic frequencies in the aerial population of spores dispersed onto the emerging autumn sown varieties can be expressed as follows:

$$f'_i = f_i \times (\sum_{j=\text{autumn}} u_{ij} \times s_j / S) \times [S + \sum_{j=\text{spring}} u_{ij} \times s_j] / w''' \quad (4)$$

where ' $j = \text{autumn}$ ' and ' $j = \text{spring}$ ' denotes summation over autumn-sown and spring-sown varieties, respectively, and the average relative fitness, w''' , of the aerial population is defined such that $\sum_i f'_i = 1$ (elaborated from Hovmøller *et al.*, 1993; Østergård and Hovmøller, 1996, unpublished).

Dynamics of gametic disequilibria

Gametic disequilibrium (linkage disequilibrium) between two virulence genes, V_x and V_y , is defined as the difference between the frequency of genotype $V_x V_y$ and the product of the single gene frequencies (Hedrick *et al.*, 1978). Using the genotype notation from above, D_{xy} can be expressed as:

$$D_{xy} = f_1 - (f_1 + f_2)(f_1 + f_3) \quad (5)$$

where f_1 , f_2 , and f_3 equal the frequencies of the two-locus genotypes $V_x V_y$, $V_x A_y$, and $A_x V_y$, respectively. Note that $(f_1 + f_2)$ and $(f_1 + f_3)$ equal the frequency of the single genes V_x and V_y , respectively. Thus, the sign of D_{xy} is positive when the genotype frequency is larger than the product of the single gene frequencies (the latter equals the expected genotype frequency in a case with random association), and negative when the genotype frequency is smaller than the product of single gene frequencies.

For a pathogen generation following random association, two- and three-locus gametic disequilibrium can be expressed relatively simply by the parameter values in the previous generation (Hovmøller and Østergård, 1991b), whereas the general expression for D is a complex function of all parameters in the previous generation (Østergård and Hovmøller, 1991).

Analysis of Virulence Survey Data

Estimating selection forces in survey areas

Virulence survey data are often published without attempt to estimate selection forces in more detail than giving information about the average distribution of resistance genes in the country (or region) considered (e.g. Wolfe, 1984; Limpert *et al.*, 1990; Munk *et al.*, 1991; Andrivon and Vallavieille-Pope, 1993). However, this may not be sufficient either for explaining the present genetic composition of a pathogen population or to predict the future gene and genotype dynamics in such population.

The simulation study by Hovmøller *et al.* (1992) showed that virulence dynamics in a biotrophic pathogen population depend on both the present selection forces (distribution of varieties with single resistance genes as well as resistance gene combinations) and the genotypic structure of the population, which may reflect selection forces in the past. In the study by Hovmøller *et al.* (1993) a more systematic approach was taken to estimate selection forces owing to host resistance genes. The distributions of potential host varieties were mapped in detail within a local barley area (approximately 3 km²), taking into account the changing distribution within and between different growing seasons in the area. This information was combined with knowledge about powdery mildew resistance genes, and combinations of these in the varieties. As a reference area, the distribution of barley varieties in a larger region around the experimental site, having a distribution different from that in the local area, was estimated on the basis of amounts of certified seed (J.O. Bagge, Copenhagen, 1989, unpublished). The selection forces were evaluated by comparisons of observed and predicted genotype (and gene) frequencies in the aerial population over 3 years. Genotypic frequencies were observed through a virulence survey comprising 11 loci, and the predictions were calculated according to Eqn 4.

Spore samples were collected in three different growing seasons, and mainly at a time when only autumn sown varieties were present, i.e. two samples in the first winter and spring, three samples in the following autumn to spring, and two samples in the third autumn. In this case, the autumn-sown barley varieties had either no resistance gene, or resistance genes for which the matching virulence genes were fixed in the aerial population. In contrast, the majority of spring-sown varieties had one, two or three resistance genes. Only non-significant differences in gene frequencies were observed for samples of airborne spores originating from the same panel of host crops, e.g. collected from autumn to spring in the same growth season, whereas highly significant differences were observed between spore samples originating from different panels of host crops, e.g. collected in autumn in different years. This emphasizes the importance of host resistance genes as selective factors, but also that

only minor changes are likely to occur when no such selection takes place (in the present case in winter).

The observed temporal changes in virulence gene frequencies (genotypes were not compared owing to their low frequencies) were generally as predicted from the model taking into account selection forces in the local area. This was the case for virulence genes subject to strong direct selection (matching resistance genes present on a relatively large area) as well as for unselected loci and for loci mainly under indirect selection (hitch-hiking). Estimation of selection forces based on the regional distribution of varieties gave results which were a poorer fit with observed dynamics (Hovmøller, 1993, unpublished).

The strength of selection for virulence depends on spore dispersal in relation to the diversity of the host plant population. Previously, powdery mildew spore dispersal studies have shown that only a small proportion of spores enter the aerial population each pathogen generation while the major part are likely to remain within the host crop (Bainbridge and Stedman, 1979). In the present case, host varieties were grown as monocultures, i.e. spores produced on one plant in a field were very likely to be dispersed to other parts of the same plant (autodeposition) or to other plants (allodeposition) of the same variety. Neither autodeposition nor allodeposition of spores within fields grown as monocultures led to additional selection for virulence. In contrast, large areas of variety mixtures would have led to increased selection for virulence within each field. This has been shown by model studies (Barrett, 1980; Østergård, 1983) and recently confirmed experimentally by Huang *et al.* (1994). A comprehensive analysis of survey data from areas with large proportions of variety mixtures can be done by extending Eqn 4 by an allodeposition parameter.

The value of virulence survey data for disease forecasts depends on the difference between the local pathogen populations, and on sampling strategy. Large differences in genetic composition, e.g. because of different selection forces in different local areas, will generally reduce the value of survey data for disease forecasts. This is the case for both sampling methods being used in the European powdery mildew virulence surveys. The mobile version of the spore trap introduced by Schwarzbach (1979), and further developed by Limpert (1985), is likely to reflect sources close to the sampling route, and samples collected from one site are likely to reflect the sources close to that site (Hovmøller *et al.*, 1995). Therefore, in virulence surveys, fixed sampling sites, as well as sampling routes, should be defined such that the source varieties are representative for the overall varietal distribution in the survey region considered.

Gametic disequilibria in pathogen populations

Gametic disequilibrium (linkage disequilibrium) has been a subject for debate in plant pathology. For example, it has been suggested that gametic

disequilibrium may reflect gene combinations in the pathogen genome that were favoured or disadvantaged by natural selection (Vanderplank, 1968; Wolfe and Barrett, 1977; Limpert and Schwarzbach, 1981; Wolfe, 1984; Welz, 1988; Zhang *et al.*, 1992).

Gametic disequilibrium may arise for many different reasons, e.g. selection, intermixture of populations with different gene frequencies, random genetic drift and mutation (Hedrick *et al.*, 1978). Therefore, as Wolfe and Knott (1982) pointed out, the interpretation of gametic disequilibrium on the basis of survey data should be done with caution. Østergård and Hovmøller (1991) showed that gametic disequilibrium between virulence genes in aerially dispersed plant pathogens can be generated as a result of selection by host resistance genes, and that the signs of two-locus gametic disequilibria could be predicted according to the use of resistance genes in host varieties. Resistance genes present mainly in different varieties are likely to generate negative gametic disequilibrium between the corresponding virulence genes, whereas resistance genes present mainly in the same variety are likely to generate positive gametic disequilibrium between the corresponding virulence genes.

The general results by Østergård and Hovmøller (1991) are illustrated in Fig. 10.3, which is based on the selection regime shown in Fig. 10.2, where each of the three resistance genes, *MLx*, *MLy* and *MLz*, are present on one-third of the area each year. Selection generates negative gametic disequilibria

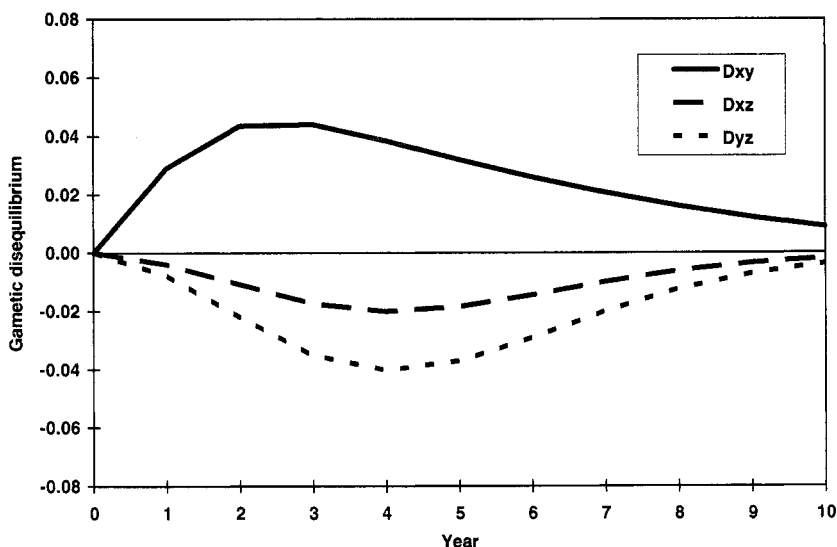


Fig. 10.3. Dynamics of two-locus gametic disequilibria over 10 years as a result of host induced selection. Host variety in field I possesses *MLx* and *MLy*, variety in field II possesses *MLz*, and variety in field III possesses none (see Fig. 10.2). The initial frequencies of the 8 three-locus genotypes equal the product of the single gene frequencies (random association), $V_x = 0.70$, $V_y = 0.40$ and $V_z = 0.10$.

between V_x and V_z as well as between V_y and V_z in the aerial population (selected for on different varieties), and a positive disequilibrium V_x and V_y (selected for on same variety). The amount of gametic disequilibrium will finally approach zero as the selected virulence gene frequencies approach fixation.

The results described above were largely in accordance with those observed in airborne mildew spore samples collected at different locations and over a number of years in Denmark, taking into account the use of resistance genes in Danish-grown barley varieties between 1980 and 1988 (Hovmøller and Østergård, 1991a). Therefore, the signs of gametic disequilibrium do not provide adequate information for drawing conclusions about the general fitness of pathogen clones based on specific combinations of virulence genes.

Dynamics of unnecessary virulence genes

Since Flor's (1953) observations on the flax rust/flax system in the north central states of the USA, and Watson and Luig's (1963) observations on the stem rust/wheat system in Australia, powdery mildew surveys have also demonstrated decreasing frequencies of virulence genes, as varieties with the matching resistance genes are withdrawn from the growing area (Grant and Archer, 1983; Wolfe, 1984; Munk *et al.*, 1991).

Wolfe (1984) and Munk *et al.* (1991) investigated long-term virulence dynamics in the UK and Denmark, respectively, where host varieties (resistance genes) replaced each other through time. Wolfe (1984) showed that the frequency of V_{a12} decreased in the UK over 17 years in each of two periods where the distribution of barley varieties with *Mla12* decreased to a minimum. Munk *et al.* (1991) analysed the frequency dynamics of nine virulence genes in Denmark between 1974 and 1989. They also observed a decrease in the frequencies of virulence genes as varieties with a matching resistance gene were withdrawn from the area. There was no general explanation for their observations, but both indirect selection (hitch-hiking) and migration were suggested as important factors. Note that in the 1960s and 1970s, powdery mildew did not generally overwinter in Denmark as autumn-sown barley was not grown, and powdery mildew was thereby reintroduced each spring by long-distance migration of spores (Hermansen *et al.*, 1978).

Inspired by some of the early survey data, Vanderplank (1968) proposed the hypothesis of reduced fitness of pathogen individuals possessing virulence genes that were unnecessary for growth and reproduction on specific varieties. Since that time the validity of his hypothesis has frequently been challenged (e.g. Leonard, 1969; Parlevliet, 1981; Grant and Archer, 1983; Bronson and Ellingboe, 1986). Grant and Archer (1983) observed a decrease in the frequency of the barley mildew virulence gene V_{a6} in England between 1969 and 1975, and calculated a coefficient of selection against this gene. During

that period, the matching resistance in barley, *Mla6*, was replaced by *Mla12* and *Mla7* combined with *MLK*. In the study by Hovmøller *et al.* (1993), the frequency of *V_{a6}* decreased significantly over 2 years. A detailed analysis of the genotype structure showed relatively strong negative gametic disequilibria between *V_{a6}* and *V_{a7}*, and between *V_{a6}* and *V_{a12}* (Hovmøller, 1992, unpublished). The strong direct selection for both *V_{a7}* and *V_{a12}* gave rise to a hitchhiking of the avirulence allele *A_{a6}*, i.e. the frequency of *V_{a6}* would be predicted to decrease in the aerial population. Likewise, the selection model developed by Hovmøller *et al.* (1992) with no fitness costs of unnecessary virulence genes confirmed that the frequency of a virulence gene is likely to decrease as the relative area with matching resistance genes becomes small. The same mechanisms may explain the observation by Grant and Archer (1983), but of course the possibility of reduced fitness of mildew clones with *V_{a6}*, when growing on varieties not possessing *Mla6*, cannot be entirely excluded.

Brown (1995) used simulation studies to analyse the fate of a virulence gene required for infection of some host varieties (necessary) and not required for infection of other varieties (unnecessary) in the same area, and another virulence gene not required for infection of any variety, the gametic disequilibrium between the two genes, and their gene frequency dynamics. The system was analysed with and without the existence of fitness costs of the two virulence genes when they were unnecessary for infection of their host varieties. The general effect of fitness costs of unnecessary virulence was to slow the rate of increase in the aerial population of the gene subject to selection by part of the host plant population, and, eventually, to remove the unnecessary gene on all host varieties. In both cases, the direction in which the frequency of the unselected gene changed depended on the presence of either a positive or a negative sign of the gametic disequilibrium between the two genes. However, as the sign for such gametic disequilibrium is difficult to predict (Østergård and Hovmøller, 1991), it may be difficult to predict the long-term dynamics of virulence genes that are unnecessary on all varieties in a certain area.

Implications for the Durability of Powdery Mildew Resistance Genes

Finally, we illustrate the implications of the previous analysis for durability of race-specific host resistance genes in a cereal growing area, i.e. the time period in which the genes provide satisfactory disease control (Johnson, 1984). The results are based on the model by Hovmøller *et al.* (1992), and extended by including the effect of using a fungicide on a variety which has become heavily diseased (see Eqn 3) (Hovmøller, 1993).

Four autumn-sown varieties designated A (*MLX*), B (*MLY*), C (*MLZ*), and D (*none*) are grown (resistance genes in parentheses), and they subsequently

replace each other during the time period considered. Two scenarios are analysed: (i) no fungicide use, and (ii) fungicide application on variety B in year 9, 10 and 11. In both cases, the initial frequencies of the three virulence genes V_x , V_y , and V_z equals 2% (arbitrary chosen), the frequencies of the 8 three-locus genotypes equal the product of the single gene frequencies, i.e. no gametic disequilibria exist, and only variety D with no resistance gene is grown. Then in year 1, variety A is introduced on 10% of the barley area. At the end of the growth season in year 1, the frequency of V_x in the aerial population has increased to 2.2%, which is calculated on the basis of Eqn 3. In the following years, the relative area of variety A increases up to 45% (year 4 and 5), after which it gradually declines to zero. Variety B is introduced in year 3 and at its maximum is grown on a slightly larger area than variety A; variety C is introduced after year 6 and has the same pattern of distribution as variety A (see Fig. 10.4).

In case (i), the frequency of the corresponding virulence gene, V_x , increases up to 66% (year 7) but decreases again to about 38% as variety A is withdrawn from the area (Fig. 10.5, solid line). The frequency of V_y increases up to 94%, and remains at a high frequency even in year 12, when variety B is withdrawn from the area. The pattern of change in frequency of V_z is much slower than that of V_x even though the matching resistance genes had been present on the same relative area.

In case (ii), a highly effective fungicide is applied on variety B (Mly) after year 8. The gene frequency dynamics are, therefore, identical in the two situations until year 9, but the decrease in frequency of V_x after variety A is

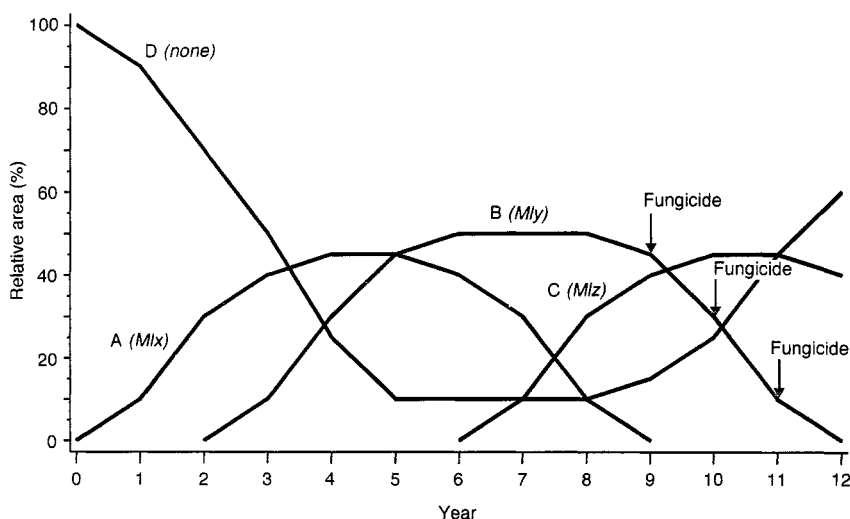


Fig. 10.4. Relative area of four barley varieties (resistance genes in parentheses) grown in a hypothetical region (for further explanation, see text).

withdrawn is now less pronounced (Fig. 10.5, dotted lines). The increase in frequency of V_y stops immediately after fungicide treatment of variety B, and in the following years, the frequency decreases to about 62%. The rate of increase in frequency of V_z , matching the resistance gene Mlz in variety C, is much larger after fungicide treatment of variety B, relative to the situation without fungicide treatment.

Three factors influenced the rate of change in gene frequencies: the relative area of varieties, the time of introduction of varieties, and fungicide treatment of varieties. The variety (resistance gene) covering the largest area resulted in the highest virulence frequency, but the resistance genes in varieties A (Mlx) and C (Mlz), which were grown on the same relative area but displaced in time, did not give rise to the same patterns of changes for the matching virulence (V_x and V_z , respectively). Fungicide treatment influenced the dynamics not only for the virulence gene matching the resistance gene in the fungicide treated variety, but also for other virulence genes in the system. In terms of durability, fungicide treatment of one variety may decrease the durability of the resistance genes in other varieties.

The explanation for the decreasing frequencies of V_x is the development of strong negative gametic disequilibria between V_x and V_y and between V_x and V_z , and a subsequent strong selection for V_y and V_z giving rise to indirect selection against V_x (hitch-hiking). The frequency of V_x does not decline to its initial value, which reflects an increasing proportion of genotypes over time

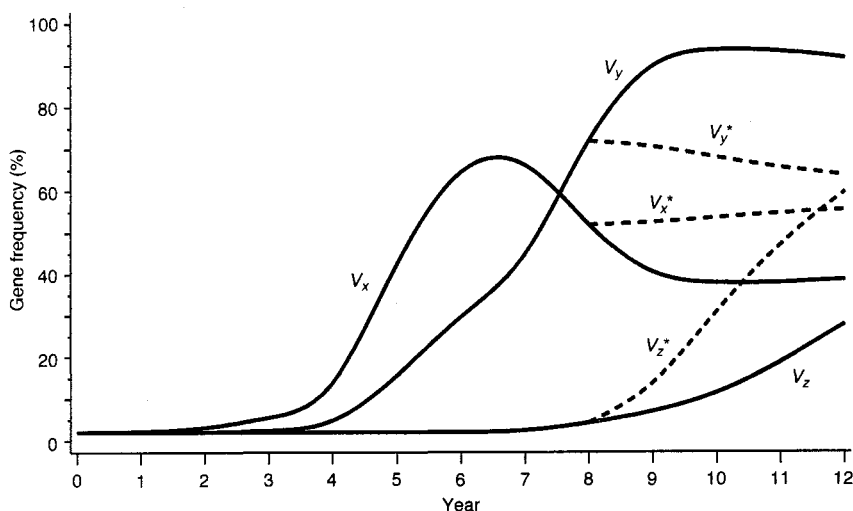


Fig. 10.5. Changes in virulence gene frequencies in an aerial mildew population present in the barley region described in Fig. 10.4. Solid lines represent population dynamics where no fungicide is used (case i), and dotted lines (and gene symbols with asterisk) represent the dynamics after fungicide treatment of variety B in years 9, 10 and 11 (case ii).

with virulence for all resistance genes present in the area. These genotypes ('super-races') will persist in the pathogen population unless the population is influenced by factors such as migration, recombination, mutation or other kinds of selection, which were not taken into account in the present example.

Concluding Remarks

These studies have demonstrated that host resistance genes are a major selective power in populations of a biotrophic and aerielly dispersed pathogen, e.g. barley powdery mildew, and that to a large extent, host induced selection determines the dynamics in such populations over the time scale considered. The results illustrate the complexity of patterns of change in the genetic composition of a pathogen population, and thereby the difficulties of *predicting* the durability of a specific disease resistance gene in an agricultural system. Therefore, long-term disease control strategies consisting of *planned* replacements of one resistance gene by another, on the basis of predicted changes in genetic composition of the pathogen population, are unlikely to be successful. Nevertheless, the results gave some indication for successful reintroduction of 'old' resistance genes in new varieties as a part of an integrated disease control strategy using different types of diversification of host varieties in time and space, and combined with a flow of new highly effective resistances, preferably polygenically based, into the ongoing breeding programmes.

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An Epidemiological Approach to Modelling the Dynamics of Gene-for-Gene Interactions

11

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Introduction

Gene-for-gene interactions between host plants and their pathogens have fascinated plant pathologists and plant breeders since the early recognition of resistance genes and the first formal statement of the gene-for-gene hypothesis by Flor (see Thompson and Burdon, 1992; Crute, 1994). The interest in these interactions continues today with the aim of full molecular characterization of gene-for-gene systems as a basis for developing improved disease control (De Wit, 1992). Mathematical modellers have long been interested in the differential effects of disease on host genotypes (e.g. see Barrett, 1988; and more recently Hamilton, 1993; Andreason and Christiansen, 1993) and models were specifically derived to consider the evolution of such genetic systems and the relationship of gene-for-gene interactions with coevolved host-pathogen associations (Leonard and Czocho, 1980; Barrett, 1986; Parlevliet, 1986; Thompson and Burdon, 1992). Even now the hypothesis continues to attract considerable controversy in terms of the assumptions made and the claimed implications at the molecular, biochemical, individual plant and population levels (Newton and Andrison, 1995).

Mathematical models of host-pathogen associations following a gene-for-gene pattern of interaction have mostly been proposed from the perspective of population genetics, with more recent attention given to some of the life-history or ecological parameters that may influence long-term dynamics. Only rarely, however, have plant disease epidemiologists contributed to such analyses through incorporating disease dynamics. This chapter outlines an approach to modelling a gene-for-gene system by integrating population

genetics, life-history and epidemiological approaches. The main characteristics of each of these approaches will first be outlined. A basic epidemiological model is then established in which a growing host population is partitioned into healthy and diseased components. From these equations is derived the basic reproductive number: the number of diseased units caused by one infectious unit in an otherwise healthy population. Host and pathogen populations are then partitioned according to a simple one-locus two-allele system in which there is specific recognition between the products of the resistance and avirulence alleles, to give a standard compatibility matrix. Population dynamics are incorporated by labelling host intrinsic growth rates and carrying capacities according to genotype; and, similarly, epidemiological parameters describing disease dynamics are labelled according to the compatible genotypic interactions.

For this genotype model, and a simplified phenotype model, results are obtained by standard qualitative analyses: for example, conditions for the persistence of the avirulence and resistance alleles or the corresponding phenotypes. An important feature is that these conditions are expressed explicitly in terms of the key epidemiological parameter, the basic reproductive number. This represents a clear advantage in interpretation and clarifies in particular the concepts of costs of virulence and resistance previously shown to be important in population genetics models. Finally, some ways of adapting the model to a range of specific circumstances will be considered, including consideration of asexual versus sexual cycles, within-season versus long-term dynamics, and disease in natural versus agricultural systems.

Models of Gene-for-Gene Systems

It is not the intention here to give an exhaustive review of the mathematical models developed to describe gene-for-gene systems. Such accounts are given elsewhere for plant diseases (Leonard and Czocho, 1980) and more recently are summarized in a broader context by Lively and Aspanius (1995). For convenience the main features of the models developed are summarized under three headings: population genetics, ecology (life history) and epidemiology.

Population genetics models

Most mathematical models of gene-for-gene systems start from a population genetics perspective. Interaction matrices of different forms, depending on the genetic assumptions made, are specified and incorporate selection coefficients or terms for relative fitness (Leonard and Czocho, 1980; Barrett, 1988). In general, experimental data on relative fitness are lacking in the literature and procedures for calculating relative fitness are not well established (Østergård, 1987). Assumptions are often made on the costs of fitness associated with host

resistance and pathogen virulence, or at least terms for these costs are included in the matrix (Leonard and Czocho, 1980; Østergård, 1982). From these matrices are specified either difference equations in discrete time or differential equations in continuous time. It is important to note that such matrices can be defined in terms of interactions other than those embodied in the gene-for-gene system but which also involve matching genetic complementarities (Barrett, 1988), e.g. matching allelic systems (Hamilton, 1993; Frank, 1993c), which differ from the usual specification of the interaction. In some cases rates of change in genotype or phenotype frequency are made frequency dependent, although there is some dispute over the nature of this frequency dependence (Barrett, 1988). Very rarely have population densities been incorporated into these models. This points to the difficulties in linking population dynamics, based on absolute fitness, and genetics, based on relative fitness. There is an increasing recognition of the need to consider absolute fitness and the importance of dealing with population density in gene-for-gene as with other genetic models (Barrett, 1988; Epperson, 1995).

Ecological (life-history) models

Absolute fitness is concerned with life history or ecological parameters such as birth and death rates, intrinsic growth rates, density-dependent factors such as carrying capacity, and the presence of competitive or other interactions. If these elements are modelled as in the classical models of population ecology – such as exponential and logistic growth and the various forms of the Lotka–Volterra equations – then expressions for absolute fitness are readily obtained using standard arguments (Roughgarden, 1979; Jeger, 1988) and from these the relative fitness of one entity (species, race, genotype) with respect to another can be calculated. However, the converse is not true: it is not usually possible to reconstruct terms for absolute fitness from estimates (usually constants) of relative fitness or selection coefficients. This underlies the caution with regard to estimating ‘parasitic’ fitness expressed by Barrett (1983). It is also unfortunate that many plant pathologists use concepts of fitness that fall outside the mainstream of population genetics (Jeger and Groth, 1985; Antonovics and Alexander, 1989).

In principle the parameters involved in absolute fitness can be incorporated into models of both the host and pathogen populations and their effect on the dynamics of a gene-for-gene system examined. A recent sequence of publications by Frank has made significant theoretical progress by including these elements. The models are derived as difference (discrete time) equations based on interaction matrices for genotypes/phenotypes, but explicitly include population sizes for both host and pathogen populations, birth and death rates, competition coefficients, and immigration and emigration (Frank, 1991a). As with the population genetics models, terms associated with costs of fitness of

resistance and virulence were written explicitly into the model formulation. It was shown that these life-history traits can be the critical element in determining the existence and persistence of polymorphisms, although the mathematical complexity increases and intuitive interpretation becomes difficult as additional elaborations are made. These elaborations include spatial aspects (Frank, 1991b), linkages between epidemiology and ecology in natural populations (Frank, 1992), both costs and benefits associated with induction of resistance to a pathogen (Frank, 1993a), multiple locus models with sexual recombination (Frank, 1993b), alternative interaction schemes to the basic gene-for-gene system (Frank, 1993c,d) and quantitative variation (Frank, 1994a,b). Although comprehensive from a population genetics/ecological perspective, and certainly in the level of mathematical and numerical analysis, the variables defined do not have immediate epidemiological interpretation (to a plant pathologist at least) especially with regard to what constitutes a pathogen individual or the unit of disease. Similarly, although the importance of the life-history parameters is shown, these are not really interpreted in epidemiological terms.

Epidemiological models

As claimed above, what is missing from most of the models concerned with gene-for-gene systems is that essential epidemiological features, as well as the life-history traits, are not accounted for. The essential features of an epidemic are the parameters describing infection or disease transmission and the subsequent progression of disease through the categories 'latent', 'infectious' and 'post-infectious'. For a fungal plant disease these categories are defined by the following lengths of time: the period prior to sporulation (latent period); and the period of sporulation (infectious period), following which sporulation is no longer possible because of colony necrosis. For a plant disease, of course, it is not normal to designate an individual host plant as the unit of disease, unlike the situation with human and animal diseases. This is one aspect of plant disease epidemics that has not been appreciated adequately by population ecologists when concerning themselves with plant diseases.

The first epidemiological model recognizing these essential features of an epidemic was the differential delay equation of Vanderplank (1963), which formed the basis of most early analysis. More recently the similarities with human and animal epidemics have been recognized (despite the problems with defining the pathogen or disease population as noted above) and models which link the rates of change of the different categories have been proposed (Jeger, 1982; May, 1990; Onstad and Kornkven, 1992; Jeger and van den Bosch, 1994). From analysis of these models (and indeed from the original Vanderplank equation) it is possible to specify a composite parameter, the 'basic reproductive number', whose value determines whether or not an

epidemic will occur. The basic reproductive number gives the number of infections that results from the introduction of one infectious unit into an otherwise healthy population during the unit's period of infectiousness. If its value is greater than one then an epidemic, in any usually accepted meaning of the term, will occur. If the value is less than one then there will not be an epidemic. The basic reproductive number has been used for a range of purposes in plant disease epidemiology, including the effect of sanitation on plant virus epidemics of perennial crops (Chan and Jeger, 1994) and in evaluating the efficacy of fungicides and fungicide mixtures (Jeger, 1995). Recently Swinton and Anderson (1995) developed an epidemiological framework for plant-pathogen interactions which they used to analyse host variation with regard to recessive resistance, but without the level of specific interaction which normally corresponds to most views of gene-for-gene systems.

As stated in the Introduction, the purpose of this chapter is to outline an approach to model gene-for-gene interactions by including epidemiology, life-history characteristics and population genetics in simplified models. In attempting this the motivation is very much in keeping with that expressed in a wider context '... of incorporating both population and evolutionary dynamics from the start' (Read *et al.*, 1995). As pointed out by others (Barrett, 1988; Swinton and Anderson, 1995), in comparable terms, there are few attempts to combine both evolutionary dynamics with realistic epidemiological assumptions. It is not the intention to give a rigorous mathematical analysis of the models developed (comparable with the models cited above), nor to give exhaustive numerical simulations, but rather to show how explicit consideration of epidemiologically meaningful variables and parameters can contribute to analysis of the long-term outcome of gene-for-gene systems, and to clarify in particular, issues relating to the pleiotropic costs of fitness associated with host resistance and pathogen virulence. No attempt is made to adapt and apply the model to any particular host-pathogen system or to an agricultural crop or natural plant population, but ways in which this could be approached are outlined.

Development of an Epidemiological Model

The starting point is a simple model for host growth which is partitioned into healthy and diseased units; the gene-for-gene interaction is then incorporated at the levels of genotype and phenotype.

Basic host model

Consider a simple logistic model of host growth in which a host population grows sigmoidally from an initial size P_0 to approach a maximum size K at arbitrarily large time t .

$$dP/dt = aP(1 - P/K) \quad (1)$$

where a is the intrinsic rate of host growth. Note that this equation makes no assumptions on whether growth is measured in terms of individuals or some measure of biomass or, for example, leaf area; or whether growth is sexual or vegetative.

Suppose further, following Jeger (1986) that the host population can be partitioned into healthy (Y) and diseased (X) units such that:

$$dY/dt = aY(1 - Y/K) - bYX/K \quad (2)$$

$$dX/dt = bYX/K - cX \quad (3)$$

where: b is the 'contact' rate (the term 'transmission' rate is also commonly used, especially where a vector is involved) between diseased and healthy units scaled by the maximum size K ; and c is the rate diseased units cease to be infectious. It is assumed that the latent period is short relative to the time span considered and thus no pre-infectious category is included. Note that the disease is considered to be of major effect in that only healthy units give rise to new host growth. Strictly it can be argued that the density-dependent term in Eqn 2 should be $1 - (Y + X)/K$ (Jeger, 1986), but this is not followed in this paper. It is also essential that healthy and diseased units are measured on the same scale. The advantage of this formulation is that it avoids the problem of defining what is meant by a pathogen individual.

By setting the derivatives equal to zero, the critical points for population densities can be found and are given by:

$$Y^* = cK/b \quad (4)$$

$$X^* = aK[1 - (c/b)]/b \quad (5)$$

where b must be greater than c , i.e. $b/c > 1$, for these to be both positive (and hence 'real') values. The quotient b/c turns out to be simply the number of diseased units caused by one diseased unit in an otherwise healthy population during its period of infectiousness, the basic reproductive number.

Note the basic similarity of Eqn 2 and 3 to the density-dependent versions of the Lotka-Volterra predator-prey equations, with of course different interpretations of the parameters, especially c . The critical points obtained can be shown to be stable equilibria (internal steady states), although the way they are approached can be oscillatory and take the form of a spiral when plotted against each other (Jeger, 1986).

Genotype model

The host population is now partitioned according to a simple one-locus two-allele system in which R represents the dominant resistance allele and r the alternative allele, which conventionally is termed the susceptibility allele. The genotypes in the healthy population are thus Y_{RR} , Y_{Rr} and Y_{rr} , which are

abbreviated to Y_R , Y_H and Y_r respectively. The pathogen population is also considered to be diploid and is partitioned similarly with respect to the dominant avirulence allele A and the virulence (again following convention) allele a . The genotypes of the disease population (to be read as diseased host units producing propagules, e.g. spores, of the respective pathogen genotype) are thus X_{AA} , X_{Aa} and X_{aa} , abbreviated to X_A , X_H and X_a .

Further suppose that specificity in the host–pathogen interaction is conditioned by the resistance–avirulence combination, which leads to the compatibility matrix in Table 11.1. The zeros in this table occur whenever the resistance and avirulence alleles interact. The numbers 1 indicate basic compatibility (lack of specific recognition) only and are not relative fitness values.

Life-history parameters are introduced by labelling the intrinsic rates of increase for the host population (Eqn 2) as a_R , a_H and a_r (using the convention above for the healthy and diseased populations) and the maximum size of each component population as K_R , K_H and K_r , and associating these with the genotypes (Table 11.2). These parameters can now be considered as selective parameters with no assumptions made as to their relative values.

Table 11.1. Compatibility matrix between host and pathogen populations in which reactions involving both the resistance (R) and avirulence (A) alleles lead to incompatibility.

		Pathogen		
		X_A	X_H	X_a
Host	Y_R	0	0	1
	Y_H	0	0	1
	Y_r	1	1	1

0 = incompatibility; 1 = compatibility; Y_R , Y_H and Y_r = genotypes Y_{RR} , Y_{Rr} and Y_{rr} , and X_A , X_H and X_a = genotypes X_{AA} , X_{Aa} and X_{aa} respectively.

Table 11.2. Matrix of selective contact rates (b) for compatible reactions between host (healthy units) and pathogen (diseased units) genotypes.

		Diseased units		
		X_A	X_H	X_a
Healthy units	Y_R	0	0	b_{Ra} (a_R , K_R)
	Y_H	0	0	b_{Ha} (a_H , K_H)
	Y_r	b_{rA} (c_A)	b_{rH} (c_H)	b_{ra} (c_a)

a = intrinsic rate of increase; K = maximum size of each component population; c = rate of removal from infectious condition; Y_R , Y_H and Y_r = genotypes Y_{RR} , Y_{Rr} and Y_{rr} , and X_A , X_H and X_a = genotypes X_{AA} , X_{Aa} and X_{aa} respectively.

The epidemiological parameters are then introduced by replacing the neutral numbers 1 in Table 11.1 by selective contact rate parameters as shown in Table 11.2; where for example b_{rA} reads the contact rate between the Y_r (homozygous susceptible) healthy population and the X_A (homozygous avirulent) diseased population. Finally, the rates of transition from the infectious to the removed condition are labelled c_A , c_H and c_a , i.e. assuming these are dependent only on the pathogen genotype. Strictly these rates also need labelling to indicate the genotype of the original healthy unit, but this does not materially alter the analysis.

Perusal of Table 11.2 shows, again, an interesting analogy with the Lotka–Volterra equations. Reading along the bottom row is analogous to the situation in which there is a more-than-one predator on a single prey; reading down the right-hand column is analogous to the situation in which there is single predator on more-than-one prey. This column is also analogous to the situation in which there is a shared disease on different host species and, for example, criteria for coexistence are sought (Holt and Pickering, 1985).

The equations for the host (healthy units) genotypes are then:

$$dY_r/dt = a_r Y_r (1 - Y/K_r) - b_{rA} Y_r X_A / K_r \quad (6)$$

$$dY_H/dt = a_H Y_H (1 - Y/K_H) - b_{Ha} Y_H X_a / K_H \quad (7)$$

$$dY_r/dt = a_r Y_r (1 - Y/K_r) - Y_r (b_{rA} X_A + b_{rH} X_H + b_{ra} X_a) / K_r \quad (8)$$

where $Y = Y_r + Y_H + Y_r$.

The equations for the pathogen (diseased units) genotypes are:

$$dX_A/dt = b_{rA} Y_r X_A / K_r - c_A X_A \quad (9)$$

$$dX_H/dt = b_{rH} Y_r X_H / K_r - c_H X_H \quad (10)$$

$$dX_a/dt = X_a [(b_{rA} Y_r / K_r) + (b_{Ha} Y_H / K_H) + (b_{ra} Y_r / K_r)] - c_a X_a \quad (11)$$

As there are now six equations involved, analysis of their properties becomes much more difficult. However some limited conclusions are possible. (These are only summarized in this section as interpretation becomes clearer when the genotype model above is reduced to the phenotype model in the next section.) If the homozygous avirulent genotype X_A^* and the heterozygote X_H^* both persist, then the critical points for the population density of the homozygous susceptible (and healthy) host population are given by:

$$Y_r^* = c_A K_r / b_{rA} = c_H K_r / b_{rH} \quad (12)$$

i.e. the basic reproductive numbers of the homozygous avirulent (b_{rA}/c_A) and the heterozygous (b_{rH}/c_H) genotypes on the homozygous susceptible host must be equal and greater than 1. Otherwise only one of the avirulent genotypes may persist. In these cases the critical point for the homozygous susceptible host population is given by its carrying capacity divided by the respective basic reproductive number in Eqn 12.

Further analysis is difficult without simplifying assumptions. If, for example, we assume that $b_{rA}/K_r = b_{Ha}/K_H$, i.e. these contact rates scaled by

their respective maximum sizes are equal, then it is possible to derive an expression for the critical point of the resistant phenotype $[(Y_R + Y_H)^*]$ in the host population. This turns out to be positive provided $b_{rA}/c_A < b_{rA}/c_A$. That is, for the resistant phenotype to persist in the host population, the basic reproductive rate on the homozygous susceptible host must be greater for the avirulent pathogen than for the virulent pathogen.

From Eqn 6 and 7, given that both Y_R^* and Y_H^* exist and are greater than zero,

$$X_A^* = a_R(K_R - Y^*)/b_{Ra} = a_H(K_H - Y^*)/b_{Ha} \quad (13)$$

For both the resistant genotypes to persist it follows that $a_R/K_R = a_H/K_H$. Otherwise only one of the resistant genotypes may persist. In these cases the critical point for the population density of the homozygous virulent genotype is given by the respective expression in Eqn 13.

Finally an expression for the sum of X_A^* and X_H^* can be derived. This turns out to be positive depending on a condition related to the ratios of the intrinsic growth rates and contact rates of the virulent genotype on the respective host genotypes. This ratio is discussed further in the simplified phenotype model.

Phenotype model

The simplifications above are made considerably clearer if the genotype matrix in Table 11.2 is reduced to a phenotype matrix by assuming a priori that $b_{Ra} = b_{Ha}$, $b_{rA} = b_{rH}$, $a_R = a_H$, $c_A = c_H$, and that there are no selective values attached to the maximum population size K (Table 11.3). In some instances this matrix could apply to two interacting haploid populations or one in which the populations are both self-fertilizing (Barrett, 1988), but this interpretation is not pursued further. In this case all the critical points are obtained explicitly and conditions for positivity (remembering that we must also have $Y < K$) are readily obtained. The density-dependence present in the host population is a strong influence on the criteria for stability.

Table 11.3. Matrix of selective contact rates (b) for compatible reactions between host (healthy units) and pathogen (diseased units) phenotypes.

		Diseased units		
		X_A	X_a	
Healthy units	Y_R	0	b_{Ra}	(a_R)
	Y_r	b_{rA}	b_{ra}	(a_r)
		(c_A)	(c_a)	

The equations for the phenotype model are:

$$dY_R/dt = a_R Y_R (1 - Y/K) - b_{Ra} Y_R X_a / K \quad (14)$$

$$dY_r/dt = a_r Y_r (1 - Y/K) - Y_r (b_{rA} X_A + b_{ra} X_a) / K \quad (15)$$

$$dX_A/dt = b_{rA} Y_r X_A / K - c_A X_A \quad (16)$$

$$dX_a/dt = X_a (b_{Ra} Y_R + b_{ra} Y_r) / K - c_a X_a \quad (17)$$

Following the same procedure as previously, we look for possibly stable steady-state or equilibrium values such that the four phenotypes persist and coexist. The critical points for population densities of the healthy host phenotypes are:

$$Y_r^* = c_A K / b_{rA} \quad (18)$$

$$Y_R^* = K [c_a - b_{ra} (c_A / b_{rA})] / b_{Ra} \quad (19)$$

which are both positive provided $b_{ra}/c_a < b_{rA}/c_A$, which is exactly the same criterion determined in the genotype model.

Thus the resistant phenotype persists if the basic reproductive number of the avirulent phenotype is greater than that of the virulent phenotype on the susceptible host. This condition can of course be interpreted as a cost of fitness for virulence, but this is not the only interpretation. It is possible that the avirulence allele, as well as serving as a recognition allele with respect to the resistance allele also acts as a pathogenicity 'factor' on the susceptible host. Whatever the interpretation of the criterion its advantage is that it is defined in terms of epidemiological parameters (and thus absolute fitness) rather than selection coefficients (and thus relative fitness).

Similarly the critical points for densities of the diseased population are:

$$X_a^* = a_R (K - Y^*) / b_{Ra} \quad (20)$$

$$X_A^* = (K - Y^*) [a_r - b_{ra} (a_R / b_{rA})] / b_{rA} \quad (21)$$

which are both positive provided $a_r/b_{ra} > a_R/b_{Ra}$, i.e. the intrinsic growth rate of the susceptible host (relative to the contact rate of the virulent phenotype on that host) is greater than the equivalent term for the resistant host. This is an equivalent expression involving growth rate ratios to that also found for the genotype model. Again, it may be possible to interpret this condition as specifying a cost of resistance to the host phenotype, but the point to be made is that it involves not only the intrinsic rate of increase of the host phenotype, but also the contact rates of the phenotypic interaction which again are epidemiological parameters. In fact there have been few studies on the costs of resistance in host phenotypes, and some have concluded that there are no such costs in particular systems that have been studied (Welz *et al.*, 1995).

Finally we need to check the conditions for which $Y^* < K$. A sufficient but not necessary condition for this is that $b_{Ra} = b_{ra}$, but this then means that (following Eqn 21) $a_r > a_R$, which is directly interpretable as a cost of resistance. Another consequence of this sufficient condition is that the basic reproductive number of the virulent phenotype is equal on the resistant and the susceptible host phenotype.

Numerical Solutions

In the case of the phenotype model a full qualitative analysis of the long-term dynamics of a gene-for-gene system can be made. The results obtained can be illustrated by solving the equations numerically and plotting trajectories of each population category with time. This is done in Figs 11.1 to 11.4 for parameter combinations in which all four phenotypes persist in the long term (i.e. the parameter combinations are such that Eqn 18 to 21 apply). Parameter values common to each numerical solution shown are the carrying capacity ($K = 1000$), initial values for the healthy host phenotypes ($Y_R = Y_r = 100$), and for the diseased host phenotypes ($X_A = X_a = 50$). In each figure the plots are made for the first 400 time units (e.g. days, corresponding approximately to a continuous 1-year period) and then for 10,000 time units (corresponding to a 25-year period). Of course the assumption of a continuous epidemic without seasonality over these periods of time is unrealistic, but serves to distinguish the different qualitative outcomes and also the differences between short- and long-term dynamics. It is also important to note that it is not possible to generalize from particular numerical solutions of the phenotype model on aspects such as long-term persistence (or extinction) or stability. These aspects can only be analysed by using qualitative techniques as in the previous section, but which are largely beyond the scope of this paper.

In Fig. 11.1 the basic reproductive number of the avirulent phenotype on the susceptible host is greater than the virulent phenotype on either the susceptible or the resistant host. There is not a great difference between the ratios of intrinsic growth rate to contact rate (see the condition following Eqn 21). Over the short term it appears that the phenotypes might be settling down to stable values, but in fact extending the time period shows damped oscillations occurring over a considerable period of time before the eventual steady-state values predicted are approached. (These can readily be checked visually from the graph). In this case the overall level of disease remains low, with the avirulent phenotype in particular remaining at extremely low levels.

In Fig. 11.2 only minor changes have been made to the parameter values and yet clearly a very different pattern of long-term dynamics occurs, with no steady-state values approached but regular and apparently stable oscillations around the values predicted in Eqn 18 to 21. Such outcomes are termed stable limit cycles and can be best visualized by plotting population values pairwise against each other, but for the phenotype model this would imply six such plots for the given outcome. In Fig. 11.3 an outcome is plotted where individual parameter values are quite different to those in Figs 11.1 and 11.2 (although the composite basic reproductive numbers are comparable) and in which host intrinsic growth rates are much higher relative to contact rates. In this case the oscillations continue into the long term and indeed diverge before eventually approaching a stable limit cycle. Again the oscillations occur around the values predicted from Eqn 18 to 21.

Finally, in Fig. 11.4 the parameter values are changed in such a way that there is greater contrast between the basic reproductive number of the avirulent (eight times higher) compared with the virulent phenotype on the susceptible host; and similarly between the growth rate ratios for the susceptible (three times higher) compared with the resistant host. In this case there are extreme fluctuations in the shorter term, but eventually these dampen out and stable steady-state values are approached. It is noteworthy that in this situation the avirulent and the resistant phenotypes can simultaneously persist

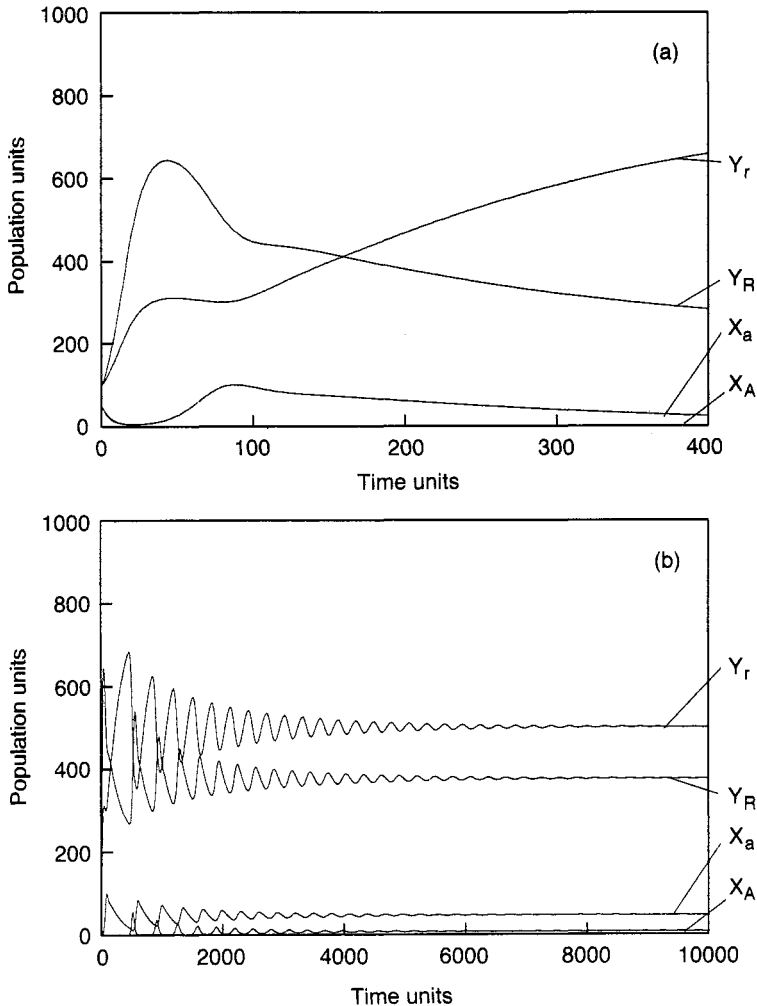


Fig. 11.1. Time plots of healthy and diseased host phenotypes over (a) 400 and (b) 10,000 time units. Parameter values are: a_R , 0.15; a_r , 0.1; b_{Ra} , 0.4; b_{rA} , 0.4; b_{ra} , 0.2; c_A , 0.2; c_a , 0.25.

in the population at high levels despite the incompatibility reaction for this combination. Again it is not possible to generalize from this on the basis of a particular numerical solution.

Interpretation and Conclusions

This model of host–pathogen dynamics following a gene-for-gene interaction can be interpreted and developed further in two main ways. First, as

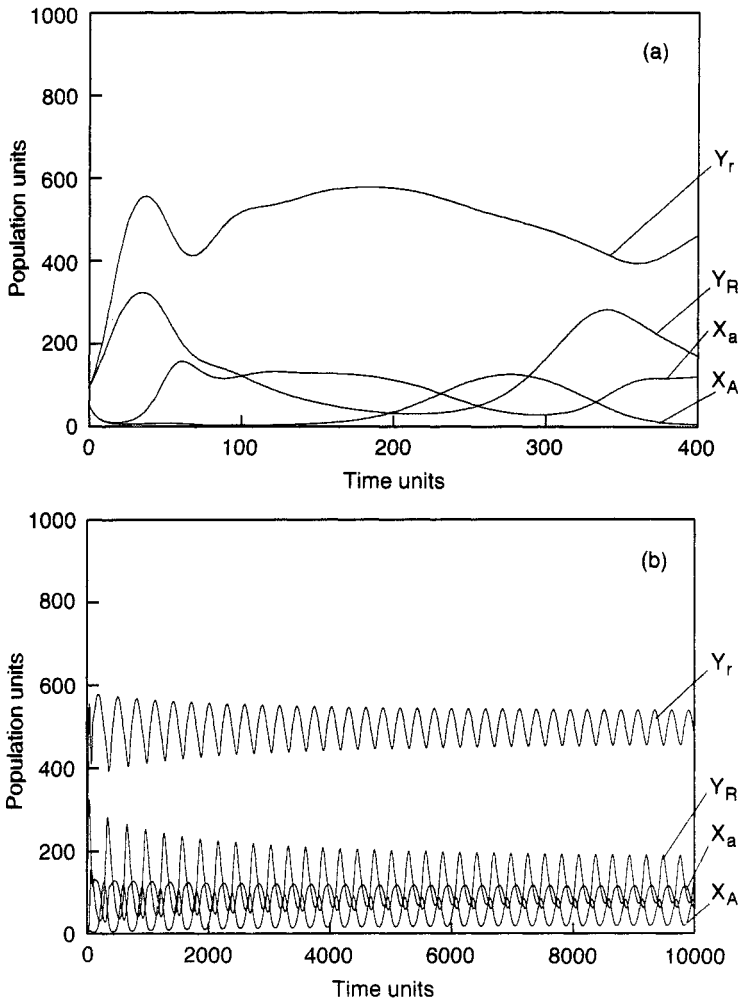


Fig. 11.2. Time plots of healthy and diseased host phenotypes over (a) 400 and (b) 10,000 time units. Parameter values (in same order as for Fig. 11.1) are: 0.1; 0.15; 0.4; 0.4; 0.4; 0.2; 0.25.

representing the long-term dynamics in populations in which both host and pathogen populations have lost the sexual cycle, initial frequencies of the genotypes are simply 'relics', and growth is clonal or vegetative. Second, and alternatively, as representing within-season epidemic dynamics with an annual or seasonal sexual cycle separating epidemics. The first interpretation is the simpler of the two and allows for the full range of qualitative analysis in determining long-term behaviour of the system, and also numerical solutions such as those presented in Figs 11.1 to 11.4. Nor is it totally unrealistic for some

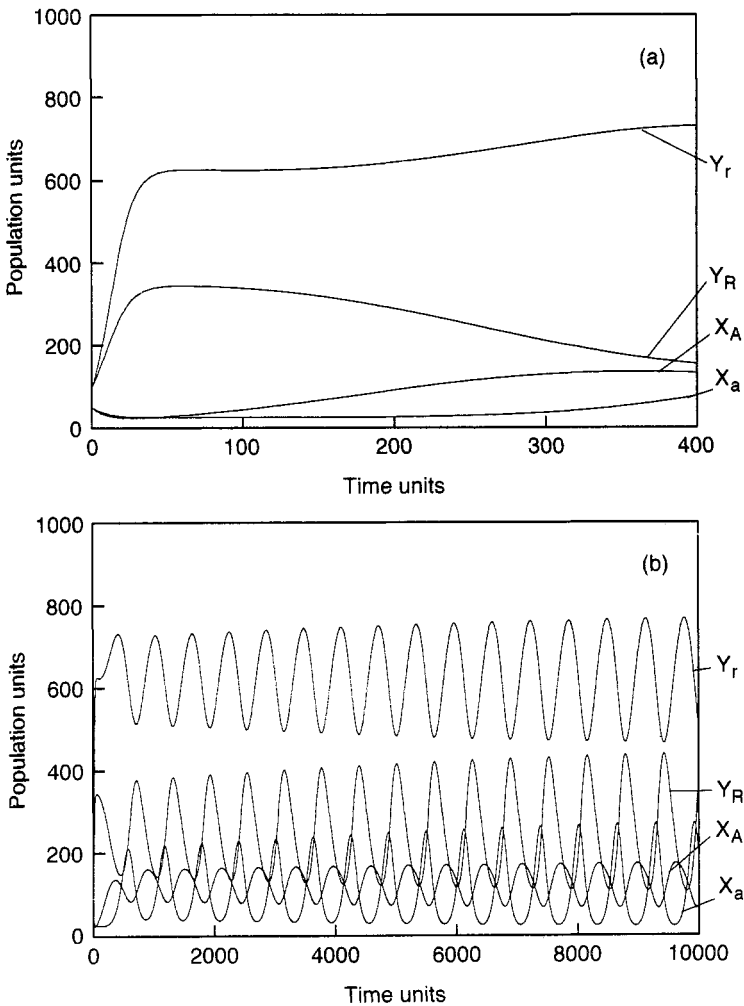


Fig. 11.3. Time plots of healthy and diseased host phenotypes over (a) 400 and (b) 10,000 time units. Parameter values (in same order as for Fig. 11.1) are: 0.1; 0.15; 0.1; 0.08; 0.08; 0.05; 0.075.

pathogen populations where the pathogenic stage is both diploid and asexual; until recently this was the situation with most populations of *Phytophthora infestans* outside of Mexico. Development of the model along these lines could apply to both natural host populations and also to those in which vegetative planting material is used for replanting.

The second interpretation, that of within-season dynamics with an interspersed sexual cycle in possibly both host and pathogen populations is perhaps more interesting and realistic for agricultural situations, although it is unlikely

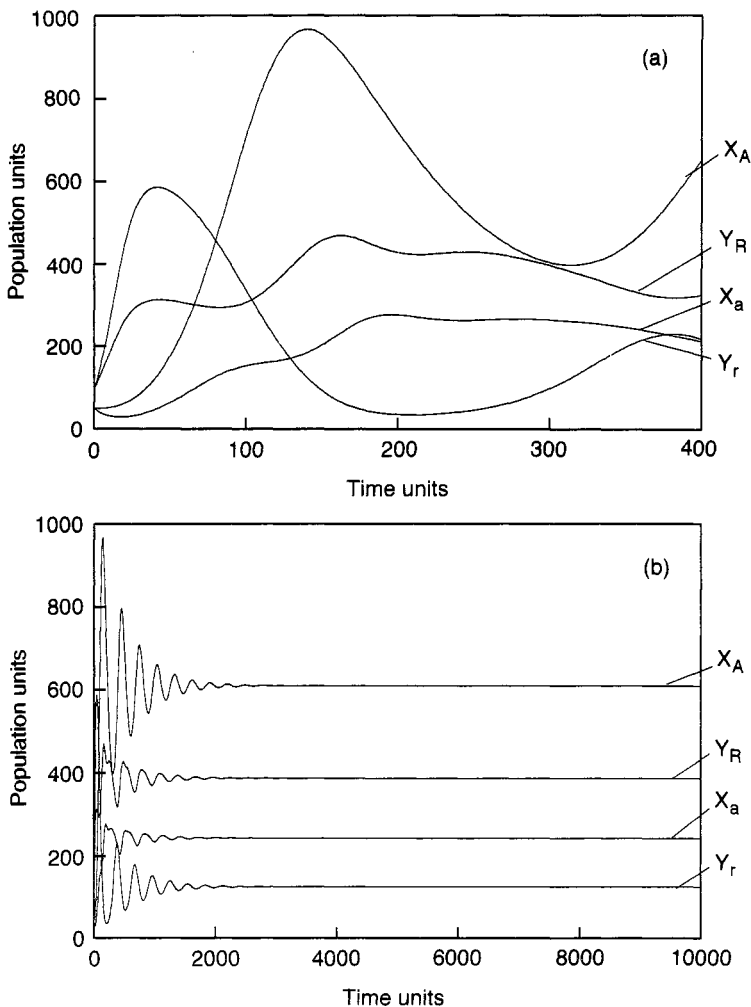


Fig. 11.4. Time plots of healthy and diseased host phenotypes over (a) 400 and (b) 10,000 time units. Parameter values (in same order as for Fig. 11.1) are: 0.1; 0.15; 0.2; 0.08; 0.1; 0.01; 0.09.

that critical points (as stable steady-state values) will be approached within a growing season (compare the short term dynamics in Figs 11.1 to 11.4). To develop the model along these lines, however, two further elaborations are necessary. First, it will be necessary to introduce sexual crossing in both host and pathogen populations at the end of the season. This procedure was followed by Frank (1993b) between generations, although this was mainly to allow for recombination and all host progeny were specified as immediately haploid. For many plant pathogenic fungi a seasonal sexual cycle, during saprophytic survival or on an alternate host, is actually a close approximation to reality. There is no need to assume random mating and Hardy–Weinberg ratios, as different degrees of inbreeding can be introduced. Second, survival (perhaps as another selective parameter or function) between the asexual pathogenic cycles would have to be introduced. Thus at the beginning of each new season's epidemic there will be new initial frequencies of each genotype/phenotype in the host and pathogen populations.

The best way of introducing these two elaborations and developing the model further would probably be through the use of discrete difference equations for both population size and frequency (Roughgarden, 1979). This procedure was followed by Doebeli (1995) in examining the relative dynamics of sexual and asexual populations, but not in the context of gene-for-gene interactions. Density- and frequency-dependence could also be incorporated in the survival functions, as could competition. The long-term dynamics (across many seasons) would then be simulated numerically. It is likely, but only hypothesized, that apparently sudden switches between qualitatively different patterns of dynamics could occur under these circumstances, i.e. periods of relative rarity of genotypes/phenotypes followed by periods of relative abundance. This of course happens as a result of the agricultural introduction of new host cultivars, but may also be a feature of natural systems in which gene-for-gene associations are found.

An alternative to introducing sexual crossing as an annual or seasonal discrete event would be to introduce continuous mating in either the genotype or phenotype model. There are several examples of the sexual and asexual cycles operating continuously during an epidemic, for example the Sigatoka leaf spot diseases of banana caused by *Mycosphaerella* spp. The continuous formulation of both clonal and sexual growth would facilitate analysis, although it is not clear how much qualitative analysis would prove possible. In a quite complex host–pathogen model, in which a diploid host was partitioned by genotype and in which there was continuous mating, it was possible to analyse population size, genotype frequency and deviations from Hardy–Weinberg equilibrium (Andreasen and Christiansen, 1993). There was, however, no partitioning of the pathogen population in this model.

It is hoped that this chapter demonstrates the usefulness of an epidemiological approach to analysing gene-for-gene interactions. Of course in some ways the framework for the model – one-locus two-alleles – could not be

simpler. Even assuming the Mendelian locus continues to be a useful basis on which to develop mathematical models, it is clear that as soon as the number of involved loci in complementary gene-for-gene systems begins to increase, then so does the range of dynamical behaviour possible (Sorarrain *et al.*, 1979; Seger, 1988; Frank, 1993b) and intuitive interpretation becomes progressively more difficult. A broader question that goes well beyond the scope of this chapter is whether the Mendelian locus does provide a valid conceptual basis for modelling gene-for-gene interactions as detailed knowledge at the molecular and functional levels increases.

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Modelling Gene Frequency Dynamics

12

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Characteristics of Natural Gene-for-Gene Systems

Gene-for-gene systems in nature

Disease resistance genes used in agricultural crops arose during long periods of coevolution of pathogens with the ancestors of current crop species (for example, see Crute, 1990). Therefore, it is not surprising that gene-for-gene relationships between resistance and virulence, which are a common feature of many diseases of cultivated crops (Day, 1974), are also found in diseases of wild plants in natural systems (Burdon, 1987). While not all of the disease resistance in wild plants can be attributed to simply inherited race-specific resistance, this type of resistance is a prominent feature of natural host-pathogen systems, particularly those involving rust and mildew fungi.

Systems of race-specific resistance and virulence in natural host-pathogen associations tend to be highly polymorphic. If they were not, we would have great difficulty in recognizing them as gene-for-gene relationships. For example, if a pathogen population were made up exclusively of the most complex race, which can overcome all known forms of race-specific resistance, the presence of resistance genes in the host would be masked. Likewise, if none of the host plants had any race-specific resistance genes, we could not distinguish pathogen races having different combinations of virulence genes. Two thoroughly studied natural host-pathogen systems, crown rust of wild oats (*Avena* spp.) and powdery mildew of groundsel (*Senecio vulgaris*), are polymorphic for large numbers of loci (Dinoor, 1977; Burdon *et al.*, 1983;

Oates *et al.*, 1983; Harry and Clarke, 1986; Burdon, 1987; Clarke *et al.*, 1990; Bevan *et al.*, 1993; Clarke, Chapter 13 this volume).

Evidence for balanced polymorphisms

In polymorphic populations, the polymorphisms may be either transient or balanced. Transient polymorphisms exist during the time it takes for a new, superior allele to increase from its first detectable levels until it completely replaces the previous allele. Balanced polymorphisms, on the other hand, persist indefinitely, because they are maintained by two opposing forces of selection that reach a balance at some equilibrium gene frequency.

Observations from natural gene-for-gene host-pathogen systems support the argument that they occur primarily as balanced polymorphisms. Briefly, the argument is as follows: if the polymorphisms were transient, we should find more diversity of resistance and more races of pathogens in areas where disease pressure is low than in areas where conditions favour severe disease development. Severe disease should cause strong selection for resistance and corresponding virulence. With transient polymorphisms, this would lead to rapid loss of diversity as genes for resistance and virulence became fixed in the host and pathogen populations. With low disease pressure, the selection would occur more slowly, so transient polymorphisms would persist longer as the gene frequencies increased slowly from barely detectable levels to fixation. Longer lasting transient polymorphisms at low disease severity would allow more polymorphic loci for resistance and virulence to occur at any given time in areas of low rather than high disease pressure.

In natural systems, however, the greatest diversity for resistance in host populations and virulence in pathogen populations is found in areas where the climate favours disease development rather than in areas where disease development is restricted (Burdon, 1987, 1991). For example, Wahl (1970) showed that resistance to crown rust (*Puccinia coronata*) races in the wild oat *Avena sterilis* is more common and more diverse in regions of Israel where the climate favours rust development than in hot dry regions where crown rust is rarely seen. Burdon *et al.* (1983) found a similar pattern for other wild oat species in Australia. Populations from northern New South Wales had significantly greater diversity of resistance phenotypes than wild oat populations in southern New South Wales where the climate is less conducive to crown rust epidemics. Oates *et al.* (1983) showed that populations of *P. coronata* from northern New South Wales had greater racial diversity than populations from southern New South Wales. Similar observations have been reported for powdery mildew of *Hordeum spontaneum* (Moseman *et al.*, 1990) and *Triticum dicoccoides* (Moseman *et al.*, 1984) in Israel.

Unlike transient polymorphisms, balanced polymorphisms tend towards equilibrium frequencies. Therefore, we should expect them to accumulate

where the host and pathogen have long associations and where disease is severe enough to impose significant selection pressure for resistance. Areas where disease pressure is high should promote more rapid accumulation of diversity for resistance and virulence than areas where disease pressure is low. As described above, this is the pattern seen in natural host–pathogen systems that have been studied extensively. Thus, observations from the natural host–pathogen systems are consistent with balanced rather than transient polymorphisms.

Frequencies of resistance and virulence

Another feature found in natural gene-for-gene systems is that virulence is much more common than resistance. Races of the pathogen are typically complex with many genes for virulence. Host plants, on the other hand, tend to have relatively few genes for race-specific resistance. These characteristics have been demonstrated most extensively for powdery mildew of *Senecio vulgaris* (Harry and Clarke, 1986; Clarke *et al.*, 1990; Bevan *et al.*, 1993; Clarke, chapter 13 this volume).

Various models describing host–pathogen coevolution in gene-for-gene interactions are consistent in showing low equilibrium frequencies of resistance in host populations and high frequencies of virulence in pathogen populations (Leonard, 1977; Frank, 1993). Thus, they fit the pattern found in extensively studied, natural gene-for-gene systems described above. The high equilibrium frequency of virulence in the models can be explained as follows. The frequency of matching virulence must be high for the disadvantage of susceptibility to be small enough to be balanced by the low cost of resistance. The frequency of resistance at equilibrium in the models is low because only then will the disadvantage of avirulence be small enough to be balanced by the cost of virulence. Thus, the conditions of relatively low frequencies of specific resistance genes and high frequencies of corresponding virulence genes are representative of host and pathogen populations at or near stable equilibria. Therefore, a key concern of the models will be to determine the conditions that can provide stable, balanced polymorphisms in coevolving gene-for-gene systems.

Modelling Selection in Gene-for-Gene Interactions

Population genetics models

Two types of host–pathogen coevolution models have been described for gene-for-gene interactions. Population genetics models account for gene frequency

dynamics while ignoring changes in population size. Ecological models, such as described by Frank (1991, 1993) and Jeger (Chapter 11 this volume) account for population size fluctuations as well as gene frequency changes within populations.

I chose the simpler population genetic model approach, because its less cumbersome mathematics allow a clearer view of how each parameter in the model affects stability of the polymorphisms. Generally, conditions that cause equilibria to be unstable in the population genetics model will also lead to instability in an analogous ecological model. An exception to this generality can occur with multiple-niche models for a patchy environment. In an environment with many separate host populations linked by low rates of migration, the rate of extinctions of host populations attacked by highly virulent pathogen populations may limit the increase of virulence. Under certain circumstances, subsequent recolonization from the host seed bank and from pathogen migration may preserve susceptibility and avirulence in the host and pathogen populations even when there is no cost of resistance or virulence. Such models represent a special case that can account for maintenance of polymorphisms under conditions that would not allow balanced polymorphisms in a single isolated host and pathogen system.

In spite of Frank's (1991) assertion that 'a joint analysis of abundance (ecology) and genetics is required to understand the maintenance of polymorphism', I believe that there is good reason to study population genetics models first before proceeding to the more complex ecological modelling. For example, as shown in the population genetics model described below, there are two ways to define cost of virulence in these models. The definition used significantly affects the stability of equilibria in the model. This is readily apparent in the population genetics model, but was not taken into account by Frank (1991, 1993) in his ecological models. Consequently, his results, while highly instructive, do not relate directly to the situation in which the cost of virulence results from reduced competitive ability rather than from an innate reduction in intrinsic rate of reproduction by the pathogen.

Modelling host fitness

The basic model described in this chapter was developed by Leonard (1977) independently of an earlier population genetics model developed by Jayakar (1970) for interactions of bacteria and bacteriophage. In Jayakar's model, a single infection by the virus kills the bacterial cell and results in replication of the virus. Leonard's model, which was developed specifically for plants and plant pathogens, treats host and pathogen fitness differently. The host is not killed by the pathogen. Instead, the pathogen reduces host fitness by reducing seed production and, consequently, the contribution of that host phenotype to the next host generation. In environments that support more severe disease,

Table 12.1. Fitness of susceptible and resistant host phenotypes in a host–pathogen model for coevolution in gene-for-gene systems.

Pathogen phenotype	Host phenotype	
	Susceptible	Resistant
Avirulent	$1 - sW_{AS}$	$1 - c - sW_{AR}$
Virulent	$1 - sW_{VS}$	$1 - c - sW_{VR}$

s = severity of disease; c = cost of resistance; W_{AS} = fitness of the avirulent pathogen phenotype on a susceptible host, etc.

the reduction in host fitness is greater. Also, in Leonard's model the reduction in host fitness by disease is proportional to the fitness of the pathogen phenotype infecting the host.

Fitness of the susceptible and resistant host phenotypes in combination with virulent and avirulent pathogen phenotypes in Leonard's model are shown in Table 12.1. In the model the parameter c represents a fitness cost of the allele for resistance. The value of c is generally assumed to be very low, because combinations of several genes for race-specific resistance in cultivated crops do not noticeably limit their yields. Attempts by Burdon and Müller (1987) and Welz *et al.* (1995) to measure fitness costs of resistance in host populations confirm that these costs must be very low if there is any cost of resistance. Harlan (1976) argued that some cost of resistance is necessary to account for the generally accepted loss of resistance in plant populations long removed from their pathogens. The parameter s represents the combined effects of all environmental factors that determine the severity of disease caused by the pathogen on a susceptible host.

The main difference between Jayakar's model and Leonard's is that in Leonard's model the loss of host fitness owing to infection by the pathogen is assumed to be proportional to an environmental parameter s for disease severity multiplied by the fitness of the pathogen in that host–pathogen phenotype combination. In Jayakar's model the loss of bacterial fitness owing to phage infection is independent of the phage phenotype's fitness except that the avirulent phage causes no loss of fitness in the resistant bacterial phenotype. Jayakar's parameter for the probability of contact between bacteria and phage is analogous to Leonard's parameter s .

Modelling pathogen fitness

Fitness of virulent and avirulent pathogen phenotypes in combination with resistant and susceptible hosts are shown in Table 12.2. Two versions of Leonard's model are considered. In the first, termed the hard selection version, there is a cost of virulence, k , that is manifested on both the susceptible and the

Table 12.2. Fitness of avirulent and virulent pathogen phenotypes in a host–pathogen model for coevolution in gene-for-gene systems.

Pathogen phenotype	Host phenotype	
	Susceptible	Resistant
<i>Hard selection model</i>		
Avirulent	1	$1 - t$
Virulent	$1 - k$	$1 - k$
<i>Competition model</i>		
Avirulent	1	$1 - t$
Virulent	$1 - k$	1

t = effectiveness of resistance; k = cost of virulence.

resistant host. In this version of the model, each additional gene for virulence is assumed to reduce the pathogen's intrinsic rate of reproduction. This is the type of cost of virulence used in Frank's (1993) ecological models. In the second version of Leonard's model, termed the competition version, the cost of virulence, k , is manifested only on susceptible plants where the virulence is unnecessary. The loss of fitness in that case can be thought of as a reduced ability of the virulent race to compete with the avirulent race when the two races co-infect susceptible plants.

Costs of virulence measured by Leonard (1969) for high population densities of *Puccinia graminis* f. sp. *avenae* (oat stem rust) in greenhouse experiments ranged from 0.1 to 0.4. Grant and Archer (1983) calculated a cost of virulence of 0.05 to 0.06 from changes in virulence frequencies in a field population of *Erysiphe graminis* f. sp. *hordei* (barley powdery mildew) in the United Kingdom.

Conditions for equilibria

From the formulae for equilibrium gene frequencies (Table 12.3) one can see that no balanced polymorphism is possible for $k = 0$ or for $s = 0$. In the competition version of the model, however, the equilibrium frequency of virulence will be greater than 0 even if $c = 0$, as long as $s > 0$ and $k > 0$. In the hard selection version of the model, the equilibrium gene frequencies for resistance and virulence are k/t and $(st - c)/st$, respectively. Thus, for the hard selection version of the model, a balanced polymorphism requires both that $k > 0$ and $c > 0$. This is a necessary condition for equilibrium also in Frank's (1993) ecological model of gene-for-gene interactions.

Equilibrium frequencies for resistance and virulence in the model over a range of values of k , the cost of virulence and s , the severity of disease, are shown in Table 12.3. For these comparisons the effectiveness of the resistance gene against the avirulent race is assumed to be complete (i.e. $t = 1$).

Table 12.3. Effects of cost of virulence, k , and disease severity, s , on equilibrium frequencies for resistance and virulence in the competition version of a host–pathogen model for coevolution in gene-for-gene systems.

Resistance $p_{eq} = k/(t + k)$		Virulence $n_{eq} = (st - c)/(st + sk)$		
k	p_{eq}	s	$n_{eq} (c = 0.02)$	$n_{eq} (c = 0.00)$
0.1	0.09	0.2	0.75	0.83
0.2	0.17	0.4	0.79	0.83
0.3	0.23	0.6	0.81	0.83
0.4	0.29	0.8	0.81	0.83
$t = 1.0$		$t = 1.0; k = 0.2$		

k = cost of virulence; c = cost of resistance; t = effectiveness of resistance; s = severity of disease.

Table 12.3 shows that the model predicts that genes for resistance in natural host populations at equilibrium will occur at moderate to low frequencies, whereas genes for virulence will usually be at high frequencies in pathogen populations at equilibrium. This means that the common pathogen races in natural gene-for-gene systems will be complex races with many genes for virulence, while host plants commonly will have relatively few resistance genes. These predictions of the model are consistent with observations for powdery mildew of groundsel (Clarke *et al.*, 1990) and crown rust of wild oats (Wahl, 1970; Burdon, 1987).

Simulation Results

Calculating host and pathogen fitness

In simulating host–pathogen coevolution, the fitness of pathogen phenotypes is calculated first. The fitness of each pathogen phenotype is determined from the values in Table 12.2 multiplied by the frequency of the indicated host phenotype during that host generation. The simulations can be run with multiple pathogen generations per host generation. Contributions of susceptible and resistant host phenotypes to the next host generation are calculated from fitness values in Table 12.1 multiplied by the frequency of the indicated pathogen phenotype, determined as described above (Leonard, 1977). If multiple pathogen generations are modelled, the frequencies of virulent and avirulent phenotypes in the last pathogen generation are used to determine the effect of disease on host fitness. Basing host fitness on the composition of the pathogen population in its last generation of increase assumes that the pathogen

population will reach maximum density near the end of the host growing season and will have its greatest impact on seed production by the host at that time.

Thus, the model can be thought of as representing an annual host plant in which each generation is derived completely from seed produced by the preceding generation of host plants. The pathogen affects host fitness by reducing production or survival of seeds per plant depending on the severity of the disease suffered by plants of each host phenotype. The simulations use difference equations rather than differential equations to represent a natural discontinuity between host generations, and a delayed impact of the pathogen on changes in phenotype frequencies in the host population through the effect of disease on seed production rather than immediate mortality of disease plants (Leonard and Czocho, 1980).

In the simulations we assume that the discontinuity between host growing seasons results in a reduction in the pathogen population density. Each year the pathogen population is assumed to start from the same low density and reach the same higher density at the end of the growing season, as in the previous year. In other words, the value of s , the disease severity parameter, is assumed to remain constant from year to year. This is an important difference between Leonard's population genetics model and Frank's (1993) ecological model of host-pathogen coevolution. In Frank's model, there is no off-season reduction in pathogen density. Instead, the pathogen population increases in density cumulatively from year to year until it drives down the host population size. Thus, in Frank's model, pathogens such as rusts and mildews with high reproductive rates tend to drive local host populations to extinction. This makes the host-pathogen interactions in his model unstable for precisely those types of diseases in which we see the greatest diversity of resistance and virulence polymorphisms in agricultural and natural systems.

Conditions for balanced polymorphisms

Results of an extensive series of simulations with Leonard's model showed that the conditions for reaching balanced polymorphisms are much more restrictive for the hard selection version of the model than for the competition version (Fig. 12.1) (Leonard, 1994). To reach equilibrium starting with virulence at mutation frequency ($< 10^{-6}$) requires a high cost of resistance when disease severity is high in the hard selection version of the model. However, equilibrium is possible in the competition version even when c , the cost of resistance, is 0. Altering the number of pathogen generations per host generation did not affect these conclusions.

Because of the evidence that natural host-pathogen systems have characteristics typical of balanced polymorphisms and because plant breeders commonly find that genes for race-specific resistance do not substantially

reduce yield potential (also see Burdon and Müller, 1987; Welz *et al.*, 1995), we reject the hard selection version of the host–pathogen model. Therefore, further discussion of host–pathogen equilibria in this chapter will concentrate on the competition version of the model.

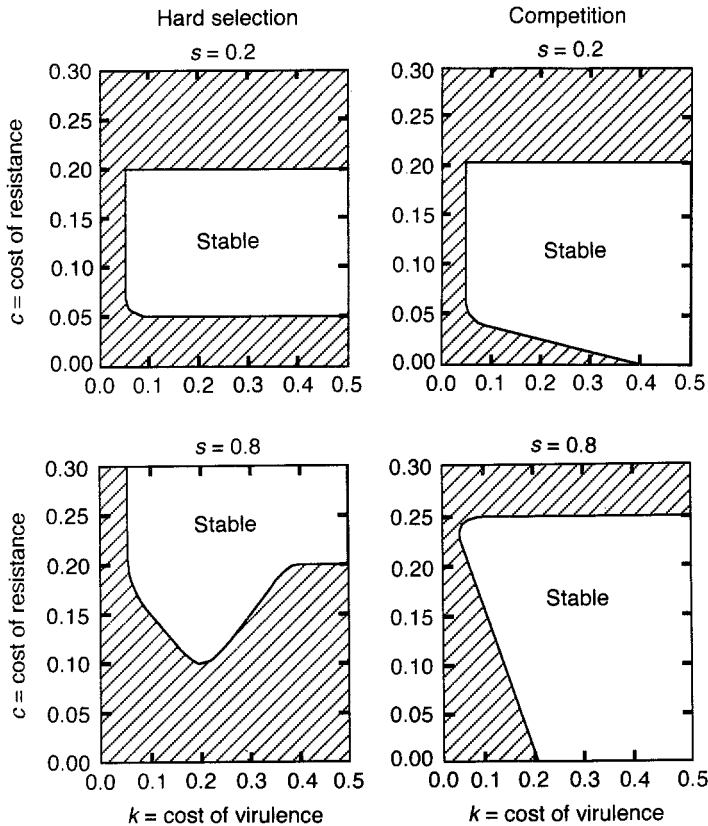


Fig. 12.1. Conditions for stable equilibria in two versions of a host–pathogen coevolution model. Combinations of parameter values in the unshaded portion of the diagrams allow frequencies of resistance and virulence in host and pathogen populations to reach equilibrium starting from a frequency of virulence as low as 10^{-6} . Combinations of parameter values in the shaded portion lead to fixation of virulence and loss of resistance if the frequency of virulence starts at 10^{-6} or less. In the model, the resistance is assumed to be completely effective ($t = 1$); s represents the severity of disease in terms of reduced fitness of susceptible host plants; c is the fitness cost of resistance; and k is the cost of virulence. In the hard selection version of the model, virulence carries a fitness cost in reduced intrinsic rate of reproduction; in the competition version, virulence has a fitness cost to the pathogen only on susceptible plants where the virulence is unnecessary.

Rate of approach to equilibrium

Simulations with the model show that the approach to equilibrium can be very slow. For example, Fig. 12.2 shows changes in frequencies of host resistance and pathogen virulence from initial frequencies of 10% through 1250 host generations. The simulations in Fig. 12.2 were run with the cost of resistance, $c = 0$; the cost of virulence, $k = 0.3$, and with the resistance completely effective, $t = 1.0$. At high disease severity, $s = 0.8$, the approach to equilibrium was faster than with $s = 0.5$, but even at $s = 0.8$ the frequency oscillations were still large after more than 1000 generations (years) for an annual host species.

Starting from mutation frequencies, it could take many thousands of years to approach within 5% of the equilibrium frequencies under these conditions. Thus, demonstrating that the equilibrium point is stable is not sufficient to account for balanced polymorphisms with reasonably consistent frequencies of resistance and virulence. If the situation illustrated in Fig. 12.2 were encountered in investigations of natural host–pathogen systems, it would have the appearance of wildly fluctuating phenotype frequencies with no obvious explanation for the fluctuations.

Effects of Pathogen Gene Flow Between Subdivided Host Populations

Pathogen gene flow in patchy environments

Individual host and pathogen populations do not exist as closed systems completely isolated from other populations of the same host and pathogen species. Host plants may be distributed in patches of varying size and host density as described for *Linum marginale* in Australia (Burdon and Jarosz, 1991), and an uneven pathogen distribution may be superimposed on the patchy host distribution. Even when there is a more uniform distribution of host plants, there may be patches with steep gradients of disease severity because of local environmental conditions. For example, both powdery mildew caused by *Erysiphe graminis* (Dinoor and Eshed, 1990) and scald caused by *Rhynchosporium secalis* (Jarosz and Burdon, 1988) are much more severe on wild barley plants growing in shade under tree canopies than on plants a few metres away in full sunlight. Therefore, it is important to explore how pathogen gene flow between patches with different disease severity may influence host–pathogen coevolution at a meta-population level.

To test the effect of pathogen gene flow between subpopulations of the host in a patchy environment, I ran the simulations for two sites with different values of s , the disease severity parameter. A designated proportion, m , of the pathogen population at each site was assumed to migrate to the other site in each generation. For simplicity, the pathogen populations in the two sites were

assumed to be of equal size, so that phenotype frequencies but not population sizes were affected by the migrations. For example, for $m = 0.05$, the pathogen population in site 1 in each generation would be generated from 95% of spores produced in site 1 and 5% of spores produced in site 2.

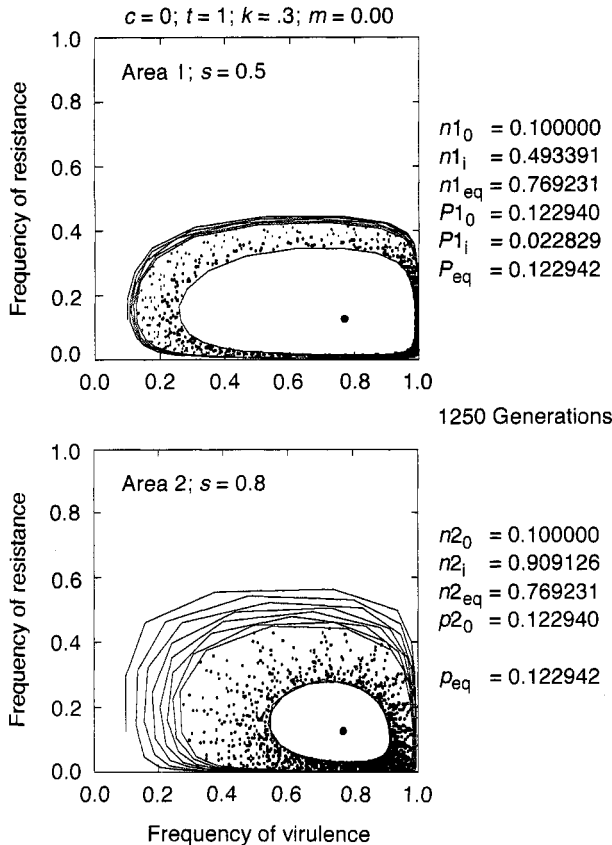


Fig. 12.2. Simulation runs for the competition version of the host–pathogen coevolution model (see text for details) for host and pathogen populations in two areas: one in which disease reduces fitness of susceptible plants by 50% and another in which disease reduces fitness of susceptible plants by 80%. In both simulation runs, the cost of resistance (c) = 0, the effectiveness of resistance (t) = 1, and the cost of unnecessary virulence (k) = 0.3. Areas 1 and 2 are assumed to be self-contained with no gene flow between them (m) = 0. Initial, final, and equilibrium frequencies of virulence (n) and resistance (p) in Areas 1 and 2 are indicated by the subscripts 0, i , and eq , respectively. The simulations were run for 1250 host generations (years) in each area. Phenotype frequency changes are plotted with lines connecting successive data points for the first 200 host generations, data points without connecting lines for the next 1000 generations, and with connecting lines for the last 50 generations to delineate the approach to equilibrium.

Impact of pathogen gene flow

Comparing Figs 12.2 and 12.3 shows that even as little as 3% gene flow between pathogen populations can greatly speed the approach to equilibrium when the host population is subdivided between areas with differing disease severity. The model parameters shown in Fig. 12.3 are exactly the same as those in Fig. 12.2 in which there is no pathogen gene flow between areas 1 and 2. Without pathogen gene flow (Fig. 12.2) much more than 1200 generations would be required to approach host and pathogen gene frequency equilibria from a starting point of 10% resistance and 10% virulence. However, with 3%

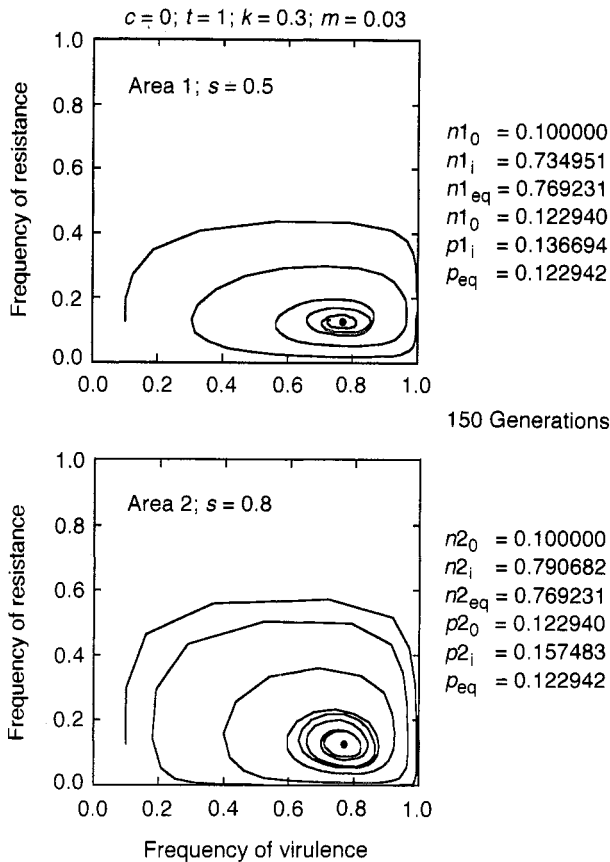


Fig. 12.3. Simulation of host–pathogen coevolution in two areas with differing disease severity. Details are as in Fig. 12.2 except that in this simulation pathogen migration between areas 1 and 2 occurs at a rate (m) of 3% of the total pathogen population. The simulation was run for 150 host generations at which time host and pathogen phenotype frequencies were close to equilibrium.

gene flow, it took only 150 generations for host and pathogen populations to approach within 5% of equilibrium values for resistance and virulence. Even when virulence in the pathogen population started at a frequency of 10^{-6} in area 2 and at 0 in area 1, the approach to equilibrium required just 250 generations (data not shown) when there was 3% migration.

The reason that pathogen migration greatly reduces the time for resistance and virulence frequencies to approach equilibrium in the model is readily apparent when one watches the changes in the frequencies in both areas during a simulation run. When disease severity differs in the two areas, the oscillations in phenotype frequencies in the two areas occur out of phase. When virulence in area 2 increases to high frequency, the frequency of virulence in area 1 tends to lag behind. During parts of the cycles, virulence is decreasing in area 2 at times when it is still increasing in area 1. The transfer of a small fraction of the pathogen population from each area to the other produces a strong damping effect on the oscillations of host and pathogen phenotype frequencies.

Adding a little pathogen gene flow to the model not only dramatically decreased the time needed to approach equilibrium in the simulations, it also expanded the range of parameter values that permit stable equilibria (Fig. 12.4). Most notably, with some pathogen gene flow it is possible to have stable equilibria in the competition version of the model with very low values of c , the cost of resistance, even when k , the cost of unnecessary virulence, is also low.

Optimal rates of gene flow for stable polymorphisms

Optimal rates of pathogen migration between two areas with different disease severity were determined by running simulations with different values of m until the frequencies of resistance and virulence reached within 5% of the equilibrium frequencies. The value of m yielding the most rapid approach to equilibrium was termed the optimal rate of gene flow for stable polymorphisms. Results presented in Tables 12.4 and 12.5 are for simulations with starting points of 1% resistance and virulence, but the number of generations to approach within 5% of equilibrium values was not much greater (less than 20% more) when the phenotype frequencies were started at 10^{-6} (data not shown).

As the difference in disease severity between areas 1 and 2 in the simulations increased, the optimal migration rate also increased and the number of generations required to approach equilibrium declined (Table 12.4). For the greatest difference in disease severity tested, $s_1 = 0.2$ and $s_2 = 0.8$, it took less than 100 host generations for the resistance and virulence frequencies to approach equilibrium closely. This result reflects the damping effect of pathogen migration on phenotype frequency oscillations. The greater the difference in disease severity between the two areas, the more out of phase are their

phenotype frequency oscillations, and the greater is the damping effect of pathogen gene flow between the areas.

When the difference between disease severities in the two areas was held constant at 0.3 in the simulations, the optimal level of pathogen migration was relatively unaffected by increasing the disease severity in both areas (Table 12.4). However, the number of host generations required to approach equilibrium increased with increasing disease severity. While it may seem counter-intuitive for greater disease pressure to delay an approach to equilibrium, this

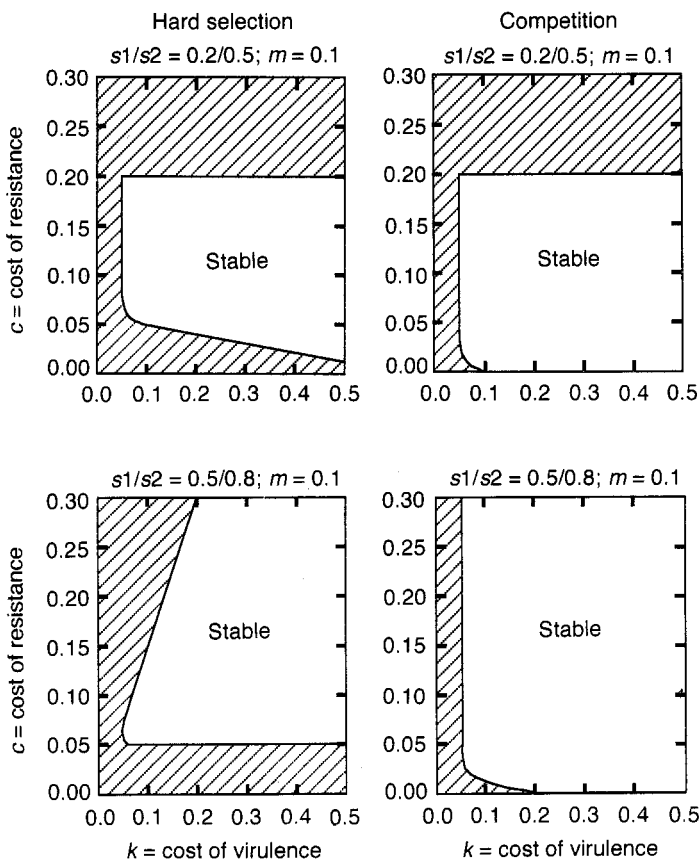


Fig. 12.4. Conditions for stable equilibria in two versions of a host–pathogen coevolution model with a patchy environment and pathogen migration between patches. See Fig. 12.1 for details. Results plotted here are from simulations involving two areas (patches) of differing disease severity ($s_1 = 0.2$, $s_2 = 0.5$; or $s_1 = 0.5$, $s_2 = 0.8$) with 10% pathogen migration between areas. Note the wider range of parameter values that allow stable equilibria with pathogen migration in a patchy environment compared with no migration or a uniform environment as in Fig. 12.1.

too is a part of the response to pathogen gene flow. When selection pressures are increased, the phenotype frequency oscillations in areas 1 and 2 tend to track each other more closely than when selection pressures are lower. The closer tracking of phenotype frequencies reduces the extent to which pathogen gene flow can damp the oscillations.

When the difference in disease severity between areas 1 and 2 was less than 0.3, optimal rates of pathogen migrations were also lower (data not shown). When there is little difference between the two areas in disease

Table 12.4. Relationship between disease severity and optimal rates of pathogen gene flow between two areas with unequal disease severity in a host–pathogen coevolution model.

Disease severity		Migration rate (m)	Generations to equilibrium ^a (g_e)
Area 1 (s_1)	Area 2 (s_2)		
0.4	0.6	0.02	530
0.3	0.7	0.06	130
0.2	0.8	0.11	55
0.2	0.5	0.05	170
0.3	0.6	0.04	230
0.4	0.7	0.04	255
0.5	0.8	0.04	260

Cost of virulence, $k = 0.2$; cost of resistance, $c = 0.02$; effectiveness of resistance, $t = 1.0$.

^aStarting point for simulation: virulence frequencies $n_1 = n_2 = 0.01$; resistance frequencies $p_1 = p_2 = 0.01$.

Table 12.5. Relationship between costs of virulence and resistance and optimal rates of pathogen gene flow between two areas with unequal disease severity in a host–pathogen coevolution model.

Cost of virulence (k)	Cost of resistance (c)	Migration rate (m)	Generations to equilibrium ^a (g_e)
0.1	0.02	0.05	760
0.2		0.04	260
0.3		0.05	160
0.4		0.07	110
0.2	0.00	0.05	290
	0.02	0.04	260
	0.04	0.03	230
	0.06	0.03	200

Disease severity in area 1, $s_1 = 0.5$; disease severity in area 2, $s_2 = 0.8$; effectiveness of resistance, $t = 1.0$.

^aStarting point for simulation: virulence frequencies $n_1 = n_2 = 0.01$; resistance frequencies $p_1 = p_2 = 0.01$.

severity, too much pathogen gene flow causes the phenotype frequency oscillations in the two areas to track each other so closely that there is little damping effect. With no difference in disease severity between the two areas, pathogen gene flow quickly causes the phenotype frequency oscillations in the two areas to become synchronized.

When there is pathogen gene flow between two areas in the model with different disease severity, the time required to approach equilibrium varied inversely with the magnitude of the costs of virulence and resistance (Table 12.5). The optimal level of pathogen migration, however, was relatively unaffected by changes in these parameters. Over all ranges of parameter values tested, the optimal rate of pathogen migration was between 0.03 and 0.07. Other simulations (data not shown) indicated that neither the rate of approach to equilibrium nor the optimal rate of pathogen migration was very sensitive to changes in effectiveness of resistance from $t = 1$ for complete resistance to $t = 0.8$ for partial resistance.

Loss of resistance or susceptibility at the subpopulation level

A total loss of resistance in one host subpopulation in a patchy environment represents a special case in which to test the effect of pathogen gene flow. The simulation illustrated in Fig. 12.5 shows that host and pathogen frequencies can approach equilibrium very rapidly with a minimum of oscillations when pathogen migration occurs between a polymorphic host population and one that is monomorphic for susceptibility. The equilibrium frequency of resistance for area 1 in Fig. 12.5 is slightly higher than the equilibrium value calculated for no pathogen migration. That is because the cost of unnecessary virulence in area 2, where the host has no resistance, reduces the level of virulence of pathogen migrants into area 1. This reduction in virulence increases the value of resistance in area 1 and, hence, raises its equilibrium frequency.

When one of the two host subpopulations in the model has no resistance, the approach to equilibrium is most rapid when disease pressure is high (Table 12.6). Even at relatively low disease pressure, however, equilibrium can be reached within 100 to 200 host generations when there is a small amount of pathogen gene flow between the areas of the two host subpopulations.

It seems significant that loss of resistance in one subpopulation of a host in the model does not destabilize the overall host-pathogen system in a patchy environment. In fact, it is not even necessary for the disease severity levels to differ in the different host subpopulations for stability to be maintained by pathogen gene flow. With the loss of resistance in area 2 in the simulations, the disease severity in area 2 became irrelevant to the damping effect of pathogen flow on oscillations of phenotype frequencies in area 1. Of course, this depends upon the relative flow of pathogen isolates from one area to the other. In real

life, that flow would depend on the number of host plants and on the average severity of infection per plant in each area. In the model simulations, the movement of pathogen spores from area 1 to area 2 was assumed to be the same as that from area 2 to area 1.

Pathogen gene flow can help maintain stability in host–pathogen systems in patchy environments also in cases in which resistance becomes fixed in one of the host subpopulations (Table 12.7). Simulations with identical parameter values to those in Table 12.6 for loss of resistance in area 2 were also run for

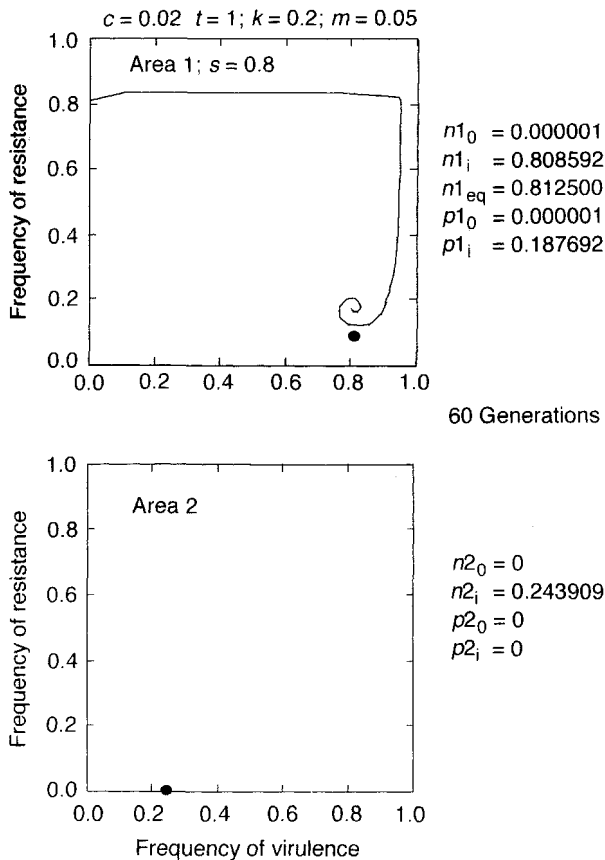


Fig. 12.5. Simulation of host–pathogen coevolution in two areas with differing disease severity. See Figs 12.2 and 12.3 for details. In this simulation, different costs of resistance (c) and virulence (k) were used, and there was a 5% rate of pathogen migration between areas 1 and 2. Also, resistance was assumed to be absent from the host population in area 2. Areas 1 and 2 are assumed to have pathogen populations of equal size. Under these conditions, the equilibrium in area 1 is stable and virulence in area 2 rises to about 0.24, a frequency at which the effect of migration from area 1 balances the cost of virulence in area 2.

fixation of resistance in area 2. The approach to equilibrium when resistance was fixed in area 2 was not so fast as when area 2 had no resistant plants. Still, the number of generations required to approach equilibrium in area 1 was much less with pathogen migration from area 2 with 100% resistant plants than it would have been with no migration between areas (compare Table 12.7 with Fig. 12.2). Although the number of host generations required to approach equilibrium increased with increasing disease severity in area 1, that effect was relatively small.

Summary

Evidence from natural host–pathogen systems is consistent with expectations for balanced rather than transient polymorphisms of resistance and virulence in gene-for-gene interactions. Greatest diversity for host resistance and pathogen virulence generally occurs in areas highly conducive to disease development. In those areas, resistance typically occurs at low frequency, whereas virulence occurs at high frequency. Low resistance and high virulence

Table 12.6. Effect of pathogen gene flow between two areas in a host–pathogen coevolution model when the host population in area 2 has no resistance.

Disease severity (s_1)	Generations to equilibrium ^a (g_e)
0.3	150
0.5	100
0.8	60

Cost of virulence, $k = 0.2$; cost of resistance, $c = 0.02$; effectiveness of resistance, $t = 1.0$; pathogen migration rate, $m = 0.05$.

^aStarting point: virulence frequencies $n_1 = 10^{-6}$, $n_2 = 0$; resistance frequencies $p_1 = 10^{-6}$, $p_2 = 0$.

Table 12.7. Effect of pathogen gene flow between two areas in a host–pathogen coevolution model when all the host plants in area 2 are resistant.

Disease severity (s_1)	Generations to equilibrium ^a (g_e)
0.3	370
0.5	390
0.8	400

Cost of virulence, $k = 0.2$; cost of resistance, $c = 0.02$; effectiveness of resistance, $t = 1.0$; pathogen migration rate, $m = 0.05$.

^aStarting point: virulence frequencies $n_1 = 10^{-6}$, $n_2 = 0$; resistance frequencies $p_1 = 10^{-6}$, $p_2 = 1$.

frequencies also are typical conditions for balanced equilibria in population genetics models of gene-for-gene interactions.

Simulations with a population genetics model for gene-for-gene interactions showed that balanced polymorphisms for resistance/susceptibility or virulence/avirulence require a fitness cost of virulence. The equilibria are more stable if the fitness cost applies only to unnecessary virulence on susceptible host plants rather than to a reduction in intrinsic rate of reproduction on all hosts. If virulence reduces the rate of pathogen reproduction on all hosts, the equilibria are unstable unless the cost of resistance is unrealistically high. When the fitness cost applies only to unnecessary virulence, equilibria may be stable even when there is no cost of resistance in the model.

Even when equilibria in the model are stable, the approach to equilibrium may take thousands of years for single isolated host and pathogen populations. However, subpopulations of host and pathogen in patchy environments rapidly come to equilibrium in the model if disease severity differs between patches and if there is a small amount of pathogen migration between host subpopulations. Differing disease severity in different patches makes frequencies of resistance and virulence oscillate out of phase in the patches, causing the oscillations to be damped strongly by pathogen gene flow between patches. With patchy environments, loss of resistance in one host subpopulation increases the stability of polymorphisms in the other subpopulations in the model rather than destabilizing them. Thus, the simple population genetics model can account for highly stable polymorphisms at the meta-population level for gene-for-gene interactions in natural host-pathogen systems.

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The Genetic Structure of Natural Pathosystems

13

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Introduction

Wild species related to crops are the commonest sources of the genes used by plant breeders to develop race-specific resistant cultivars. However, little is known about how the resistance these genes determine functions in the survival strategy of any wild host, although such knowledge could indicate how the genes could be used best to obtain long lasting protection in crops. It has been suggested (e.g. Day, 1974) that the genes may not operate in a race-specific manner in the wild host and that their race-specific effects in crops could result from their transfer without many of the controlling elements that normally regulate their activity. If the latter is the case, then the role of the resistance determined by these genes in the survival strategy of wild hosts will not be understood without investigation of wild plant pathosystems. This chapter reviews some of these studies with reference to the occurrence and role of race-specific resistance.

The Occurrence of Race-Specific Resistance in Wild Plant Species

Unambiguous proof that resistance genes may operate in a race-specific manner in wild species requires an analysis of wild plant pathosystems which have coevolved by natural selection with little if any influence from any related crop pathosystem. Investigations of wild relatives of crops in areas where the crops

are also grown are unlikely to provide unambiguous proof, because the genetic structure of the wild plant pathosystem and therefore the function of the resistance genes could be significantly altered by the adjacent crop pathosystem.

Although many studies have indicated that race-specific resistance does occur in wild plant pathosystems, there are few which provide unambiguous proof. One of the earliest of these studies was that by Dinoor (1977) in Israel on resistance in two wild oat species, *Avena sterilis* and *A. barbata*, to one of their parasites, *P. coronata* f. sp. *avenae*. Both studies showed the marked differential interactions between oat lines and rust isolates which are characteristic of race-specific resistance. The two wild oats are among the most common plants in the native flora in Israel, but the cultivated oat is only grown as a very minor crop (A. Dinoor, Warwick, UK, 1995, personal communication). Thus the cultivated crop pathosystem is unlikely to have had much impact on the evolution of the two wild oat/crown rust pathosystems.

Clearly, however, the best evidence for the operation of race-specific resistance in wild plant pathosystems comes from studies on pathosystems which have no direct relationship with any crop or no direct relationship with any crop grown within the region of study, e.g. the studies in Britain on the *Senecio vulgaris*/*Erysiphe fischeri* pathosystem (Harry and Clarke, 1986; Bevan *et al.*, 1993a,b) and the studies in Australia on the *Glycine canescens*/*Phakopsora pachyrhizi* (Burdon and Speer, 1984; Burdon, 1987), and the *Linum marginale*/*Melampsora lini* (Burdon and Jarosz, 1991, 1992) pathosystems.

The *Senecio vulgaris* (groundsel)/*E. fischeri* pathosystem is an ideal system for establishing the occurrence of race-specific resistance and for investigating its role in the survival strategy of the host for several reasons. First, the host is a monocarpic, strongly inbreeding, short cycling annual weed of disturbed land. It is a particularly common weed of cultivated land especially land involved in horticulture. It produces seed freely (strictly, single-seeded achenes) and although the seed retains its viability for little more than 2 years, collections of plant lines can be maintained as pure lines over long periods through the production of fresh seed at appropriate intervals. Thus abundant, genetically uniform, plant material can be produced easily for experiment. Groundsel can be found infected with the mildew at almost any time of the year, although heavy infections occur most commonly in the summer and autumn months. The mildew, *E. fischeri*, although related taxonomically to the aggregate species *E. cichoracearum*, is considered distinct enough to be a separate species (Blumer, 1967). Its sexual stage has been recorded on the continent, notably in Switzerland and Sweden (Blumer, 1967; Junell, 1967), but asexual reproduction by means of conidia has only ever been recorded in Britain. Its host range includes a number of *Senecio* spp. (Clarke *et al.*, 1990), but none of them are related to a crop species. However, even though the *S. vulgaris*/*E. fischeri* pathosystem is not related to a crop pathosystem, it is possible to argue that it has been, and still is, subject to considerable human influence by virtue of the fact that it is a weed of disturbed land and particularly of cultivated land. Thus

the population size of *S. vulgaris* and therefore of its mildew, *E. fischeri*, is likely to have changed substantially with the spread of cultivated land over the last 5000 years or so since the dawn of agriculture in Europe. The populations of both host and parasite will have fluctuated widely over the years owing to changes in crops and cropping practices and also, in the short term, as the result of cultivations and other cropping practices during the growing season. However, none of these events are likely to have direct effects on the coevolving genetic systems of the host and parasite populations in the way that related crop pathosystems would be expected to and natural selection is likely to be the main driving force for the coevolution of the interaction.

Marked differential interactions between isolates of *E. fischeri* and lines of *S. vulgaris*, both with respect to complete resistance, and to incomplete resistance have been found (Harry and Clarke, 1986; Bevan *et al.*, 1993a,b). However, the interactions between some groundsel lines and mildew isolates are more varied and complex than those generally described for crop pathosystems and therefore more difficult to interpret. For convenience in recording, infection types have generally been categorized into a number of discrete classes, although in reality infection type is a continuous variable ranging from little or no spore germination to prolific growth and sporulation. The categories used were generally: type 0 (germination only) and type 1 (very limited mycelium development with very limited sporulation), both requiring the light microscope to categorize them, and types 2 to 4 or 6 (mycelial development from a level just visible to the naked eye, with sporulation of around 50 conidia mm⁻² of leaf surface, to extensive mildew development leading to the production of up to about 1000 conidia mm⁻² of leaf surface) which were categorized using the unaided eye.

For most mildew isolates, but particularly for the more aggressive ones (Table 13.1), the frequency distribution of infection type is generally strongly bimodal, because of the relative infrequency of the low to intermediate types (types 1 to 2). A distinction can thus be drawn between the very low infection types (0 to 1), which clearly reflect complete or near complete resistance and the higher infection types (2 to 6) which reflect decreasing levels of partial or incomplete resistance (Harry and Clarke, 1987). This distinction was supported by a genetic analysis of the host determinants of infection type. Thus F₂ progenies from crosses made between plants expressing infection types 0 or 1 and plants expressing infection types 2 to 6 to an isolate, segregated to give simple Mendelian ratios, commonly 3 : 1 but also 15 : 1, low : high infection types (Harry and Clarke, 1987; Campbell, 1990). These ratios are consistent with the involvement of an oligogenic system. In contrast, the F₂ progenies of crosses made between plants expressing infection type 2 and plants expressing infection types 3 to 6 to an isolate, showed continuous segregation indicative of the involvement of polygenic systems (Harry and Clarke, 1987). The genetic determinants of complete or near complete resistance and those of partial or incomplete resistance are thus quite distinct and similar to the genetic systems

Table 13.1. (a) Frequencies of infection type and mean infection type for 24 mildew isolates on 50 lines of *Senecio vulgaris*. (b). Avirulence genes postulated to be present in each mildew isolate which match one or more of the 14 resistance genes postulated to be present among the 50 host lines (adapted from Bevan *et al.*, 1993a).

	Mildew isolate																							
	N11	N9	G2	N1	G1	G3	G12	G9	N7	G7	G10	N12	N4	N10	G6	N6	N8	G4	N2	G11	N5	N3	G5	G8
(a) Infection type																								
0	10	11	12	11	11	12	5	7	9	12	11	9	10	9	7	9	9	10	9	9	9	9	10	7
1	7	2	6	3	5	0	9	4	5	0	0	4	4	4	1	2	4	1	3	1	2	0	0	2
2	10	9	8	5	6	5	5	4	4	5	3	3	0	2	7	2	0	1	0	0	0	3	0	0
3	17	12	5	14	5	9	8	9	4	4	7	3	1	1	3	2	1	3	2	2	1	0	1	1
4	4	7	4	5	4	7	5	6	3	6	3	4	10	7	7	4	1	2	3	3	2	2	0	2
5	1	5	7	9	8	7	3	9	6	3	9	10	6	8	9	7	9	6	6	6	2	1	4	3
6	1	4	8	3	11	10	15	11	19	20	17	17	19	19	16	24	26	27	27	29	34	35	35	35
Mean	2.10	2.66	2.72	2.76	3.08	3.20	3.36	3.48	3.62	3.62	3.72	3.74	3.82	3.86	3.86	4.14	4.24	4.24	4.26	4.46	4.54	4.58	4.66	4.76
(b) Postulated avirulence genes	A7	A6	A2	A4	A1	A10	A3	A8	A9	A10	A11	A6	A5	A6	A10	A11	A10	A11	A10	A11	A11	A11	A11	A11
						A11				A11	A12						A13	A11	A12	A11	A14	A12		A14
						A12				A12	A14						A14	A12	A14	A12		A13		
						A14				A14								A13		A13				
																		A14						

which have been found to control similar forms of resistance in crop plants (Crute, 1985).

Many of the isolate/line tests gave an infection type, either low or high, with small or no variance (Bevan *et al.*, 1993a), making it possible to classify the interactions unambiguously as incompatible (infection types 0 to 1) or compatible (infection types 2 to 6). Such cases produced many examples of the differential interactions characteristic of race-specific resistance. However, in a significant number of cases it was not possible to classify the interactions unambiguously (Bevan *et al.*, 1993a). For example, interactions which gave infection types intermediate between type 1 and type 2, i.e. where mildew development was much higher than that of infection type 1 but still less than that of infection type 2. These infection types were most commonly produced by some of the less aggressive isolates (to the left in Table 13.1), and may therefore result as much from the lack of aggression of the isolate as the incomplete expression of an *R*-gene. Classification was also problematic in cases where the different leaves of a line, and even different parts of the same leaf, expressed different infection types to an isolate, including both low (resistant) and high (susceptible) infection types. Some of this latter variability could be attributed to changes in resistance as the plant or its tissues matured, and evidence for both seedling and adult tissue resistance was found, as well as for environmentally (temperature) induced changes in resistance (Bevan *et al.*, 1993c).

Despite the difficulty of characterizing some host line/mildew isolate interactions as unambiguously incompatible or compatible, it is possible to assign specific resistance phenotypes and specific avirulence phenotypes respectively to many lines and isolates, either partially through a visual assessment of data sets or more comprehensively using the computer program developed by Sutherland (1986). Such analyses indicate that surprisingly large numbers of specific resistance factors may be required even in quite small samples of the host population to explain the interactions; a minimum of 14 factors were required to explain the interactions between a random sample of 24 mildew isolates and 50 host lines. The ease with which new specific resistance can still be found in the 50 line set clearly indicates a very complex system of race-specific resistance in groundsel. Furthermore, the studies on wild oats, and wild flax, although less detailed, indicate that the complexity of race-specific resistance found in *S. vulgaris* in relation to *E. fischeri* system is not unique.

Other studies, although on wild species related to crops grown within the region of study, also indicate that race-specific resistance may be widespread in wild species, e.g. the race-specific resistance shown in *Lactuca serriola* (prickly lettuce) to *Bremia lactucae* (Crute, 1990) and in the common weed grass, *Elymus repens*, to *Erysiphe graminis* (unpublished results). In the latter case, a small collection of 25 lines of *Elymus repens* revealed the differential reactions characteristic of race-specific resistance to inoculation with two isolates of *Erysiphe graminis* obtained from the host. The molecular biologists' plant, *Arabidopsis*

thaliana, has also been shown to possess race-specific resistance to the downy mildews, *Peronospora parasitica* and *Albugo candida* (Crute *et al.*, 1994) and the bacterial pathogens *Pseudomonas syringae* (Staskawicz *et al.*, 1994) and *Xanthomonas campestris* pv. *campestris* (Tsuji *et al.*, 1991). Clearly there is good evidence to indicate that race-specific resistance can be a common coevolutionary outcome of interactions between wild plants and at least some of their parasites.

The Genetic Basis of Race-Specific Interactions in Wild Plant Pathosystems

A complete genetic analysis of race-specific resistance in the host and avirulence/virulence in the parasite, to confirm the occurrence of a gene-for-gene interaction, has not been carried out for any wild plant pathosystem. However, the pattern of interactions between *S. vulgaris* lines and *E. fischeri* isolates is entirely consistent with a gene-for-gene interaction (Harry and Clarke, 1986) and a partial analysis of resistance in *S. vulgaris* to *E. fischeri* supports this contention (Harry and Clarke, 1987; Campbell, 1990). The specific resistance factors identified in several lines of *S. vulgaris* by their specific reactions to different mildew races were found to be determined by a series of major genes, one gene for each resistance factor. Only one resistance gene was identified at each locus and so, apart from the allele for susceptibility, no evidence for multiple allelism at any locus was found. Most of the loci appeared to be in the same linkage group, either in one large gene family or in several gene families. A small number of resistance genes were located in a separate linkage group, either located more distantly on the same chromosome, or, since *S. vulgaris* is a tetraploid species, on the chromosome homoeologous to the one on which the large linkage group is located. In most cases resistance was dominant over susceptibility but a number of crosses yielded a susceptible F₁, indicating that susceptibility could be the dominant trait. However, segregation in the F₂ generation of such crosses indicated that the apparent reversal of dominance was due to the co-segregation of dominant non-allelic suppressors of the resistant genes (Campbell, 1990; Clarke *et al.*, 1990).

The Distributions of Specific Resistance and Specific Virulence in Populations of Wild Plants and Their Parasites

An understanding of the role of race-specific resistance in the survival strategy of wild hosts requires knowledge of the distribution of resistance phenotypes and virulence phenotypes in interacting host-parasite populations. Since such distributions could be drastically affected by interference from related cropping

systems, it is essential, and even more so than for the simple demonstration of race-specific resistance, that the wild plant pathosystem studied is one which is unrelated to any crop pathosystem within the region so that the coevolution of both host and parasite is entirely the result of natural selection. It is also important that the pathosystem is the result of a relatively long period of coevolution and is not in the early stages of coevolution, such as the recently established associations between *S. vulgaris* and the rust, *Puccinia lagenophorae*, (Wilson and Henderson, 1966) or the blister rust, *Albugo* sp., attributed to *A. tragopogonis* (Preece and Francis, 1987). Few of the wild pathosystems which have been studied meet these criteria but, as indicated earlier, one of the closest is the *S. vulgaris*/*E. fischeri* pathosystem (Harry and Clarke, 1986; Bevan *et al.*, 1993a,b). The following account is based largely on that pathosystem.

The distribution of resistance factors in populations of *S. vulgaris*

In the first survey of groundsel for race-specific resistance, plant collections, usually of ten lines each, were made at about 25 sites, mostly in Scotland, but also from a few other sites elsewhere in the British Isles (Harry and Clarke, 1986). Specific resistance to one or more of five different virulence phenotypes of the mildew (the lines of about half the samples were also tested with a further three isolates making eight in total) was found in some lines in all the samples. About 50% of the plants collected around Glasgow, the location from which all eight mildew isolates were obtained, were resistant to one or more of these isolates, while all of the plants in a number of samples collected some distance from Glasgow possessed some resistance. The most resistant sample was collected from around Perth, about 100 km north east of Glasgow. All of the ten plants in this sample possessed some specific resistance, ranging from resistance to two isolates (two plants) to resistance to six isolates (two plants) and eight of the ten plants were of different resistance phenotypes. Just over half of the total collection (250 lines) were resistant to one or more of the five mildew isolates with the rest being susceptible to all. However, very few (ten out of 250 lines) possessed resistance to all five isolates and only two were resistant to all eight.

While the early study indicated high levels of heterogeneity, both within and between groundsel populations with respect to specific resistance, it gave no indication of the way the distribution of resistance in the host population might be related to the structure of the mildew population with which it is in contact. A more detailed study was therefore undertaken of specific resistance in two populations of groundsel, one in Glasgow, Scotland and one about 480 km south at Wellesbourne, in the Midlands of England (Bevan *et al.*, 1993b), to a random collection of five mildew isolates obtained from each population. At both Glasgow and Wellesbourne, between 80 and 90% of the

plants were susceptible to all ten of the races of mildew used, but a proportion were resistant to one or other of the races. The frequency of resistance to each race in each population ranged from 1 to 10%, with the exception of resistance to one of the Glasgow races which was present in 37% of the plants sampled at Wellesbourne. Although both the Glasgow and Wellesbourne populations tended to be dominated by one or two resistance phenotypes, they were highly heterogeneous for resistance when the less frequent resistance phenotypes were considered. This was particularly evident at Wellesbourne, where ten different resistance phenotypes, including the phenotype susceptible to all races, were recorded amongst 75 plants growing within an area of 1 m².

A significantly higher proportion of the plants collected from Wellesbourne than from Glasgow contained specific resistance to one or other of the ten mildew races. Furthermore, both the Glasgow and the Wellesbourne populations, but particularly the Wellesbourne population, contained more specific resistance to the mildew races obtained from the Glasgow population than to the races obtained from the Wellesbourne population. Clearly the Wellesbourne population is more heterogeneous for specific resistance than the Glasgow population, but whether this reflects the effects of higher infection pressures by the mildew or of other factors is not known.

The heterogeneity found with respect to specific resistance, particularly within the plants located in 1 m² at Wellesbourne, is surprising because *S. vulgaris* is a strongly inbreeding species with less than 0.1% cross-pollination (Hull, 1974). Although the achenes are windborne, the bulk of production tends to land close to the parent plant (Sheldon and Burrows, 1973). Thus the population would be expected to have a much more clonal structure than appears to be the case with respect to resistance phenotype, with near neighbours showing a high degree of genetic identity through common ancestry. It would be interesting to analyse the population for unselected markers, to determine if the heterogeneity for specific resistance matches the underlying clonality of the population, or if specific resistance is a much more variable trait imposed on a more widespread clonal system.

Variation in the mildew population

In contrast to the host, *E. fischeri* is a more difficult organism to handle and maintain and so our knowledge of the mildew is derived largely from detailed analyses of a limited number of races (Harry and Clarke, 1986; Bevan *et al.*, 1993a). Apart from the information obtained from a limited study using mildew gardens (Bevan *et al.*, 1993a), little is known about the frequencies of different avirulence/virulence phenotypes in any population.

All mildew isolates whose virulence phenotype has been examined in detail have been found to have complex virulence. All eight races used in the first study possessed combinations of virulence effective against all but one of

the specific resistance factors postulated to be present in the plant lines on which they were tested (Harry and Clarke, 1986). Each race thus differed from the other seven by the possession of a unique avirulence factor. Eighteen different races were identified among the random sample of 24 mildew isolates (Table 13.1) used in the later studies of Bevan *et al.* (1993a). Of these 18 races, 14 possessed virulence for all but one of the 14 different resistance factors postulated to be present among the lines on which they were tested, while the other ten were only slightly more restricted in their host range, the most restricted race having virulence for nine of the resistance factors and avirulence for six (Table 13.1). No mildew isolate (about 100 examined) has been found with virulence for all host lines, but because each isolate has such complex virulence most are able to attack between 80 and 90% of their host's population. Clearly the race-specific resistance deployed in the host population provides little protection against each mildew race.

An attempt was made to assess the frequency with which virulence for certain resistance phenotypes occurred in the mildew population at Wellesbourne, by exposing plant lines with different numbers of resistance factors to natural infection in mildew gardens (Bevan *et al.*, 1993a). Lines with the highest number of known specific resistance factors were generally the last to become infected, indicating that mildew races with virulence for lines with few or no specific resistance factors were more common than races with virulence for lines with the most specific resistance. The presence of high numbers of resistance factors in a line thus appears to delay the onset of infection and so afford some protection against the mildew. However, apart from one line which has remained totally free from mildew infection despite regular exposure to the chance of natural infection for nearly 20 years in Glasgow, all plant lines which showed total resistance to all isolates with which they were tested in the laboratory did eventually become infected when exposed to natural infection in the field. The study with the 'mildew garden' at Wellesbourne was limited to an extent, because none of the lines with high levels of specific resistance were from the Wellesbourne host population; in some cases they were from groundsel populations 500 km or more distant. Thus the low frequency of virulence for these lines in the Wellesbourne mildew population could be due to the fact that some of the specific resistance factors in the lines were not represented in the local host population and therefore the mildew population was not specifically adapted to them. It is, however, interesting to note that virulence was present in the mildew population, albeit perhaps at a low level, for all but the one totally resistant line.

Studies by Dinor and Eshed (1987) showed similar complexity among isolates of *E. graminis* f. sp. *hordei* collected from wild barley (*Hordeum spontaneum*) at different locations in Israel. In these studies, 350 mildew isolates were tested on 36 barley (*H. vulgare*) cultivars, each cultivar possessing one or more of the resistance genes commonly used in barley cultivars in Europe. The mildew isolates possessing virulence for all of the R-genes present in the

36 cultivars made up the largest fraction of the isolates collected from all but one site and all isolates were, to some extent, of complex virulence. The related variation in the host population was not investigated, but the study indicates that the complexity in virulence phenotype found in *E. fischeri* is not unique to this species but is probably a common coevolutionary outcome of plant-parasite interactions. Of course, the level of variation within the population of an organism will depend upon its breeding system. High levels of variation would be expected in *E. graminis* in Israel because of the importance of the sexual stage in the life cycle. On the other hand, *E. fischeri* appears to reproduce entirely asexually in Britain and it is not clear how, without a sexual stage, the high level of variation in this species is generated and maintained.

The role of race-specific resistance in the survival strategy of S. vulgaris

The knowledge gained of the race-specific resistance structure of the groundsel population and of the avirulence/virulence structure of the mildew population does allow some conclusions to be drawn of the probable significance of race-specific resistance in the survival strategy of groundsel. Clearly, there is extreme heterogeneity for race-specific resistance in *S. vulgaris* populations, but this resistance can only provide limited protection because the complex virulence of *E. fischeri* ensures that each isolate is able to attack 80 to 90% or more of the host population. Some groundsel plants do have high numbers of undefeated specific resistance, so they are resistant to many virulence phenotypes of the mildew population (Harry and Clarke, 1986) and when exposed to natural infection in 'mildew gardens' tend to remain free from infection longer than lines with little or no specific resistance (Bevan *et al.*, 1993b). Such plants would be expected to have a selective advantage over lines with few or no undefeated specific resistance, although this is clearly not the case since they are in the minority in the population. For example, in a random sample of 46 groundsel lines collected in Glasgow, only one line was resistant to all ten virulence phenotypes of the mildew with which they were tested, but 44 lines were susceptible to all, or all but one, of them (Bevan *et al.*, 1993b). A further sample of 47 groundsel lines collected at Wellesbourne, contained two lines resistant to all ten virulence phenotypes but 42 susceptible to all, or all but one, of them (Bevan *et al.*, 1993b). Whether or not high levels of specific resistance impose a cost on fitness is currently under investigation.

The Enigma of Race-Specific Resistance

The studies reviewed clearly show that race-specific resistance is a feature of wild plant species and is not simply an artefact of crop plants. Race-specific

resistance would be expected to play some role in a host's survival strategy, but the extent of this role will obviously depend upon the rates of response of the two interacting gene systems, the *R*-genes in the host and the *Av*-genes in the parasite. There is ample evidence from crop pathosystems to show that the introduction of new *R*-genes in crops, particularly those conferring resistance to airborne parasites of the aerial surfaces, can confer total resistance to all elements of the parasite population. However, such resistance generally leads to the rapid appearance of new virulence phenotypes of the parasite and rapid changes in the virulence structure of the parasite population, often within a few years of the introduction of the new *R*-gene (Wolfe and McDermott, 1994). The *Av*-genes appear to be highly mutable. Unfortunately, there is little or no evidence to indicate how frequently mutations for new resistance occur and how rapidly they can spread through the population. Some evidence of rates of mutation and how race-specific resistance may evolve in wild pathosystems by natural selection could be obtained from studies of the recently established associations between *S. vulgaris* and two parasites, the rust, *P. lagenophorae*, and the blister rust, *Albugo* sp. *P. lagenophorae*, is now widespread and common on *S. vulgaris* throughout Britain, but it was not recorded in Europe until the early 1960s (Wilson and Henderson, 1966). The new *Albugo* sp., is equally common and widespread on *S. vulgaris*, but its association with this host is even more recent, being first recorded in Britain in 1978 (Preece and Francis, 1987). Preliminary studies indicate that *S. vulgaris* has not evolved race-specific resistance to either parasite in the short period since the associations established. First, a small sample of 50 lines of groundsel that had been shown to include a wide range of specific-resistance phenotypes to *E. fischeri* (Harry and Clarke, 1986; Bevan *et al.*, 1993a) were all found to be susceptible to a single pustule isolate of *P. lagenophorae*. Furthermore, when exposed to natural infection in the field, these same lines all became relatively heavily infected by both *P. lagenophorae* and the *Albugo* sp., yet showed clear evidence of specific resistance to *E. fischeri*. It seems unlikely that those 50 plants of *S. vulgaris* would possess so many specific resistance factors to *E. fischeri* yet none for specific resistance to either *P. lagenophorae* or the *Albugo* sp. if such resistance were present.

Clearly, studies on these new associations could provide valuable clues regarding the rates of development of resistance genes and the evolution of race-specific resistance in coevolving host-parasite systems. However, the evidence suggests that avirulence genes mutate to virulence more rapidly than new resistance genes arise and this, together with the greater fecundity of polycyclic parasites of the aerial surfaces of plants than their hosts, is likely to ensure that any benefit from new resistance genes will be short lived. Despite the fact that most lines of *S. vulgaris* are susceptible to infection by common elements of the mildew population, the host remains a common species. It is thus likely that genetic systems have evolved in the host in response to repeated infection with virulent isolates and that these systems play a greater role than race-specific resistance in the survival strategy of groundsel.

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The Evolution of Gene-for-Gene Interactions in Natural Pathosystems

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Introduction

Race-specific resistance, the most obvious manifestation of gene-for-gene interactions between plants and their pathogens, is just one of a variety of ways whereby plants protect themselves from pathogen attack. Indeed, the evolutionary response of plants to pathogens is governed by the combined effects of a wide range of morphological, biochemical and physiological responses. These may lead to avoidance of the pathogen in time, inhibition or prevention of pathogen establishment, reductions in the rate of pathogen development or lastly, increased tolerance of pathogen presence. In the plant, genetic control of these resistance mechanisms ranges from the action of single genes to the action of many genes simultaneously; in the pathogen, pathogenicity and aggressiveness are inherited independently and are similarly, typically controlled by single and many genes, respectively. It is the interaction of all these genes in both host and pathogen that, modified by environmental effects, generates the realized resistance profile of the plant.

The relative importance of different resistance mechanisms in natural communities is a topic of considerable debate. However, the range of individual mechanisms and expressions of resistance that occur in plants are so diverse that while it is important to recognize their existence, it is impossible to consider them all simultaneously in anything but a most superficial manner. Resistance based on morphological characters that exclude pathogens (for example, protective bud scales and glumes) or on the action of many genes that reduce the rate of pathogen development and its ultimate fecundity (quantitative resistance) occurs in all plant species. Gene-for-gene systems, on the other

hand, are not universally distributed but many of the elements of such interactions – resistance conferred by single genes with major phenotypic effects and tightly coupled interactions between host lines and specific pathogen genotypes – have now been found in a wide array of host–pathogen associations (Thompson and Burdon, 1992; Burdon *et al.*, 1996).

In the past, thinking about the evolution of gene-for-gene interactions has been dominated by the role of single gene resistance in agriculture. Theoretical models developed in that era predicted cyclical polymorphisms of resistance and virulence resulting from frequency-dependent selective processes acting against the most common local host genotype, and maintained through time by fitness costs associated with resistance and virulence (Person, 1966; Leonard, 1977; Groth and Person, 1977; Levin, 1983). More recently, the artificiality of excluding the ecological setting of an interaction from considerations of its genetic dynamics has become increasingly clear (Barrett, 1980; May and Anderson, 1983) and the first models that explicitly treat both these factors in plant–pathogen, gene-for-gene models are now appearing (Frank, 1992, 1993; Leonard, Chapter 12 this volume).

The Metapopulation as a Framework for Coevolution

The majority of plant species growing in natural communities occur as a patchwork of local demes of varying size, temporal predicability and spatial distribution, that are subject to varying amounts of interpopulational dispersal. Each deme is established by colonists from other local populations and, in turn, may give rise to new colonies before eventually becoming extinct. Much long-standing theoretical work has examined the evolutionary consequences for plants of this distribution of individuals into a network of local demes (Wright 1943; Kimura and Weiss, 1964) or metapopulations. In contrast, the direct and indirect (via host populations) effects of these basic ecological features on the populations of pathogens that plants may harbour has received little attention. In pathogens, population subdivision can be even more marked than in their hosts. Population sizes may show vastly greater amplitudes with precipitous crashes following near exponential increases in numbers. The importance of these effects and their ramifications can be illustrated by examining the demographic and genetic consequences of the interaction of just two very basic characteristics of all pathogen life histories – the demographic cycle and off-season survival mechanisms.

Demographic considerations

Pathogen survival and extinction

All populations of pathogens go through demographic ‘boom and bust’ cycles characterized by periods of low numbers, followed by rapid population

expansion to epidemic levels that ultimately cannot be sustained and are hence followed by population collapses. Although examples of such fluctuations exist in natural host–pathogen associations, their broader significance for the coevolution of hosts and pathogens has largely been missed. In this context, it is the speed, intensity and duration of the ‘crash’ phase that is of particular significance. Such population crashes are typically associated with harsh environmental conditions that reduce or locally eliminate susceptible host tissue. This has the effect of reducing the effective size of the host population as a resource for the pathogen. How pathogens cope with the survival consequences of these combined effects (rapidly falling population numbers and ‘safe’ sites) has the potential to have a marked influence on local and regional pathogen population size and genetic structure. Indeed, as shown in Fig. 14.1, the size of host populations may interact with pathogen off-season survival mechanisms to define plant population sizes that effectively act as thresholds for pathogen survival.

As the size of host populations fall, both the total amount of host tissue and the amount of living susceptible tissue also falls. For pathogens with efficient means of off-season survival, for example sclerotia or teliospores, this may present little difficulty and the probability of survival of such pathogens within

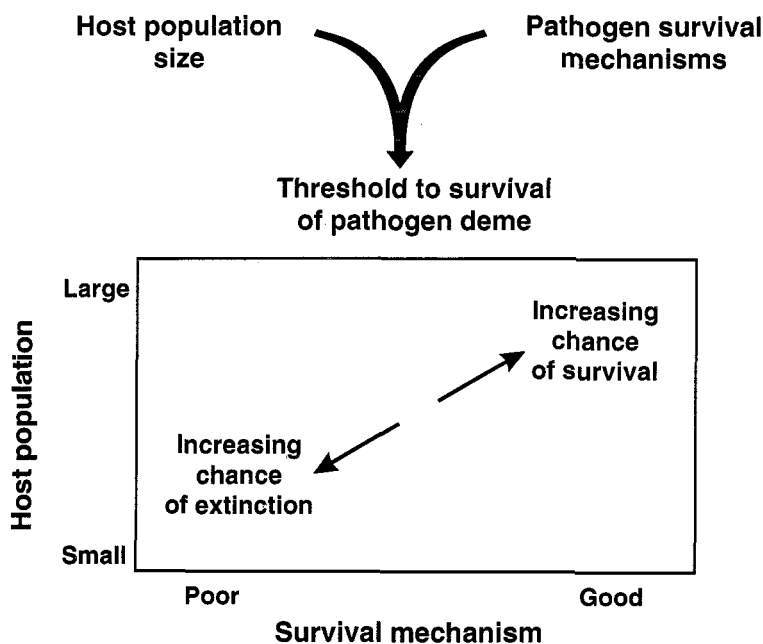


Fig. 14.1. Schematic diagram showing how the size of host populations may interact with the efficiency of pathogen off-season survival mechanisms to generate minimum host population sizes necessary for the local maintenance of the pathogen.

even small populations may be substantially greater than zero. However, as the efficiency of pathogen off-season survival mechanisms decline, there is an increasing chance of local extinction, particularly when host populations are small (bottom left corner, Fig. 14.1).

Empirical evidence for the existence of such thresholds is limited although over a single annual transition, populations of the pathogen *Melampsora lini* occurring on small demes of *Linum marginale* (< 100 plants) showed a greater probability of extinction than did populations occurring in medium to large host patches (> 100 plants) (Burdon and Jarosz, 1992). In that interaction, the pathogen has no special off-season survival mechanisms. In contrast, systemic infections of *Viscaria vulgaris* by *Ustilago violacea* are highly protected and Jennersten and his colleagues (1983) found that only patches of less than ten plants were free of disease. More recently, details of the link between host population size and pathogen survival have been teased out by a 4-year study of the association between *Filipendula ulmaria* and its rust pathogen, *Triphragmium ulmariae* (Burdon *et al.*, 1995). A non-linear relationship was found between the size of 129 host populations and the survival of the pathogen (Fig. 14.2), such that the probability of pathogen extinction in the off-season was high in host populations smaller than approximately 500 plants in size while extinction was rare in those exceeding 1000 plants.

As with most interactions between hosts and pathogens, environmental factors may affect the strictness of pathogen survival thresholds. In the *F. ulmaria*/*T. ulmariae* association, more sheltered sites (curves 1 and 2; Fig. 14.2), where teliospores of the pathogen were less likely to be stripped away by storm action, consistently showed evidence of lower thresholds than more exposed sites. Equally, year-to-year fluctuations in the harshness of the physical environment at a single site may also cause variation in the size of survival thresholds. Thus in an experimental study involving field plantings of hundreds of individuals of *L. marginale* infected with *M. lini*, Burdon and Elmqvist (1996) showed that at one site the probability of survival of individual infections varied from 0.007 to 0.200 and 0.009 over three consecutive winter seasons. These values imply host population sizes ranging from 13 to 425 for a 95% or greater probability of pathogen survival. From studies of this type, and particularly that of the *F. ulmaria*/*T. ulmariae* association, estimates of annual pathogen extinction rates in local populations (4 to 10%) clearly indicate the dynamic nature of many host-pathogen interactions.

Temporal and spatial fluctuations in pathogen populations

One expectation of host-pathogen associations acting in a metapopulation framework is evidence of temporal and spatial fluctuations with component demes showing varying degrees of asynchrony in pathogen incidence and abundance. Clearly, local pathogen extinction will result in marked temporal and spatial variation in the occurrence of pathogen populations. However,

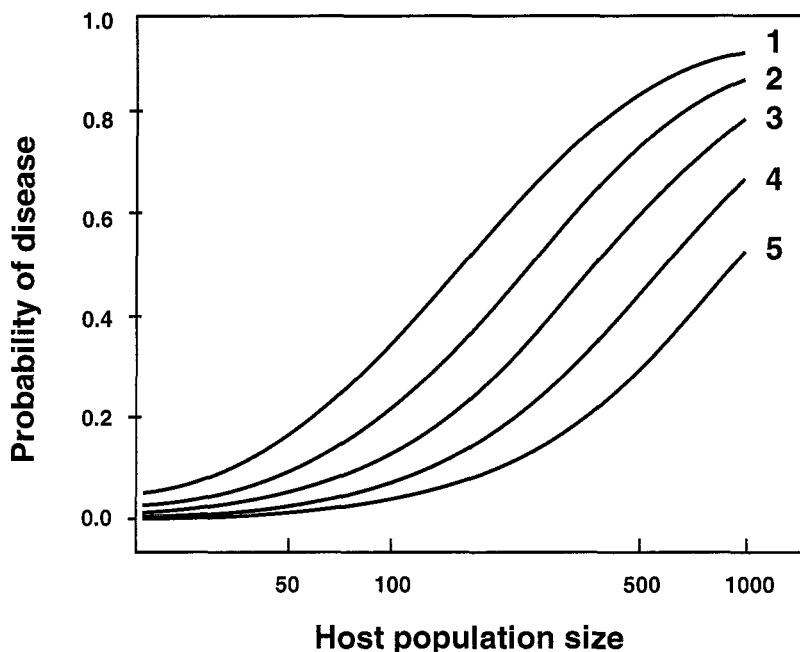


Fig. 14.2. The relationship between host population size and the probability of survival of populations of the rust pathogen, *Triphragmium ulmariae*, in 129 populations of *Filipendula ulmaria*. The five curves indicate the change in intensity of the relationship as sites become less favourable for the pathogen (1 to 5, decreasing favourability for the pathogen; from Burdon *et al.*, 1995).

even without such extreme outcomes, disease levels are frequently found to fluctuate greatly from year to year and place to place. Over a 10-year period, three epidemics of *M. lini* (> 10% leaf area diseased) occurred in one intensively monitored population of *L. marginale*. These epidemics occurred in years 1, 4 and 8; in other years, disease incidence ranged from a total absence of the pathogen, through trace occurrences (one or two pustules on 200 plants) to disease peaks of 2% of all tissue infected. Similarly, within a single year, four populations of the same plant occurring in a 1.5 km² area showed disease incidence values ranging from 0 to 26% (Jarosz and Burdon, 1992; J.J. Burdon, unpublished data). A long-term study of the rust pathogen, *Uromyces valerianae*, on 30 populations of *Valeriana salina* occurring on small islets off the southern Bothnian coast of central Sweden has documented even greater within-season spatial fluctuations in disease levels. There, infection levels in adjacent populations separated by only 100 m ranged from 1 to 100% (L. Ericson, Umeå, 1995, unpublished data).

Spatial fluctuations in disease intensity result in ever-shifting probabilities as to which populations will be most likely to contribute to the migrant pool at

any given time. To date, it has not been possible to monitor the spatial origin of migrant pathogen propagules in any of the host–pathogen associations discussed here. Indeed, evidence of migration *per se* is only easily obtained when previously pathogen-free host populations become infected. However, migration must also occur into infected populations although such migrants will only be detected when they differ sufficiently from resident pathotypes and subsequently increase in frequency to a detectable level.

The degree to which individual demes are connected is of vital significance in determining whether they are truly acting as a metapopulation or simply as a highly dispersed single population. An indirect way in which some light can be thrown on this question is to test for relationships between the disease status of all host demes within a local area, the size of those demes and their proximity to one another (Hanski, 1994). This has been done for each of the 4 years of the *F. ulmaria*/*T. ulmariae* study and the interrelationship found to be complex (Fig. 14.3; Burdon *et al.*, 1995). In all years there was a strong tendency for large infected host populations to be nearer to other infected populations than were disease-free ones. However, as the size of host populations fell, the likelihood that they would support a pathogen population declined dramatically regardless of the proximity of infected neighbours. Equally, as the degree of physical

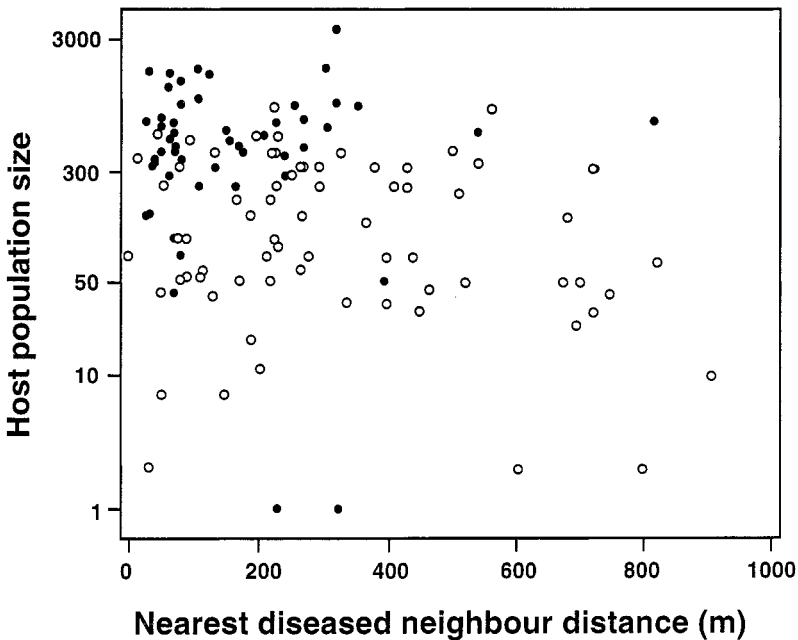


Fig. 14.3. Interrelationships between the size of host populations, their disease status and their proximity to the nearest neighbouring disease population (from Burdon *et al.*, 1995). Closed circles represent infected populations.

isolation increased, the probability of infection of either large or small neighbouring populations declined, although isolated pockets of infection did occur.

Genetic considerations

The preceding discussion indicates that the dynamic behaviour of real pathogen populations conforms to the basic expectations of the metapopulation model – that is, pathogen populations tend to be patchy, spasmodic and temporally variable showing large amplitudes in size, relatively frequent local extinctions and complex degrees of asynchrony between neighbours. From a genetic point of view, these sorts of dynamics raise expectations of marked fluctuations and changes in the structure of individual pathogen populations as a consequence of the combined action of genetic drift, extinction and subsequent recolonization, limited interdemographic migration and local selection.

Pathogen population structure

To date, all the evidence available to test these expectations comes from detailed studies of the rust pathogen, *M. lini*, found on a series of populations of *L. marginale* in southern Australia. The pathotype structure of individual pathogen populations in this interaction shows considerable temporal and spatial variation. Detailed examination of these patterns in two populations only 300 m apart at Kiandra, New South Wales provide a clear picture of this variation (Fig. 14.4; J.J. Burdon, unpublished data). In both populations, and in most years, many different pathotypes of *M. lini* were detected. However, only six of these occurred at sufficient frequency to be considered separately. At site P1, the structure of the pathogen population varied markedly between years, with only pathotype K being present for the entire period. During the same time, pathotypes A, E and N occurred in some years and not in others. Moreover, even when present in consecutive years, the frequency of all pathotypes showed considerable fluctuation. In contrast, the pathogen population at the main Kiandra site was dominated in all years by pathotype A. However, there, the disappearance of particular pathotypes during the off-season transitions 1988 to 1989 (H) and 1990 to 1991 (AL/AR) is strongly suggestive of the chance consequences of genetic drift during the intervening winter seasons.

The full extent to which drift appears to generate year-to-year fluctuations in the presence or absence of particular pathotypes is shown in Fig. 14.5, where the frequency of all four of these common pathotypes in nine populations is plotted against their frequency in the following year. Data points arrayed along the x-axis represent examples of pathotypes that were present in one year and absent in the next. In contrast, data points arranged along the y-axis provide examples of a pathotype appearing within a population after

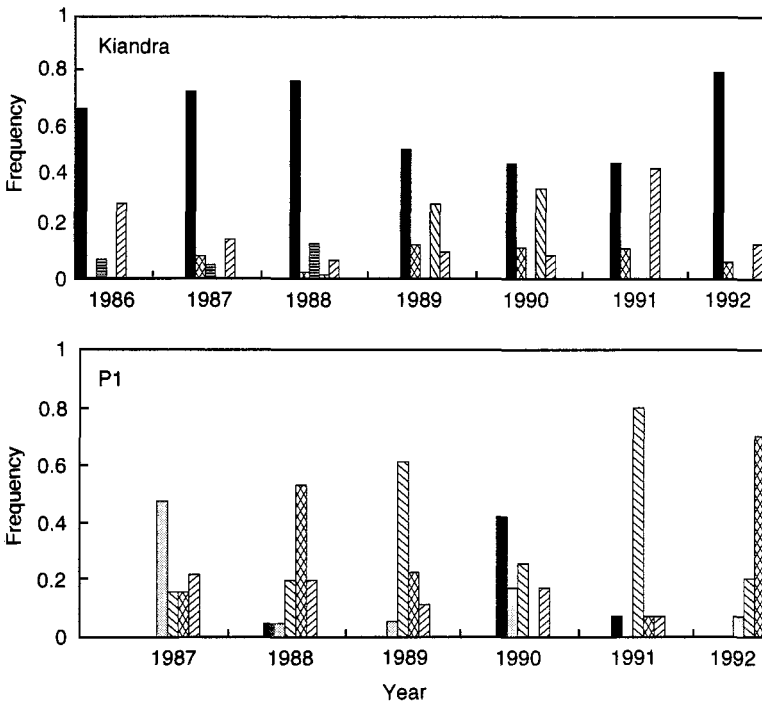


Fig. 14.4. Pathotype structure of two populations of *Melampsora lini* occurring on *Linum marginale* at Kiandra, New South Wales. Individual frequencies are shown left to right for: Kiandra population – pathotypes A (■), H (▨), N (▩) and the AL/AR complex (▧); P1 population – pathotypes A (■), E (▨), K (▩) and N (▧). Individually, the frequency of all other pathotypes (▧) was low. These have been combined into a single value.

being absent the previous year. The proportion of paired year comparisons showing putative evidence of drift is 19.5%. This value is undoubtedly inflated by situations in which particular pathotype frequencies have fallen below a detectable level but not actually gone extinct. However, it clearly indicates the potential importance of this consequence of host population fragmentation for the genetic structure of individual pathogen demes.

The data shown in Figs 14.4 and 14.5 also provide evidence for two other features which would be expected to influence local pathogen population structure under the metapopulation model. In a metapopulation system where pathogen population extinction occurs, migration events leading to recolonization and to diversification of existing pathogen populations must also take place. Because of the presence of a resident pathogen population, identification of the latter type of event will be difficult. However, one example is provided by the appearance of pathotypes AL and AR in the intensively monitored

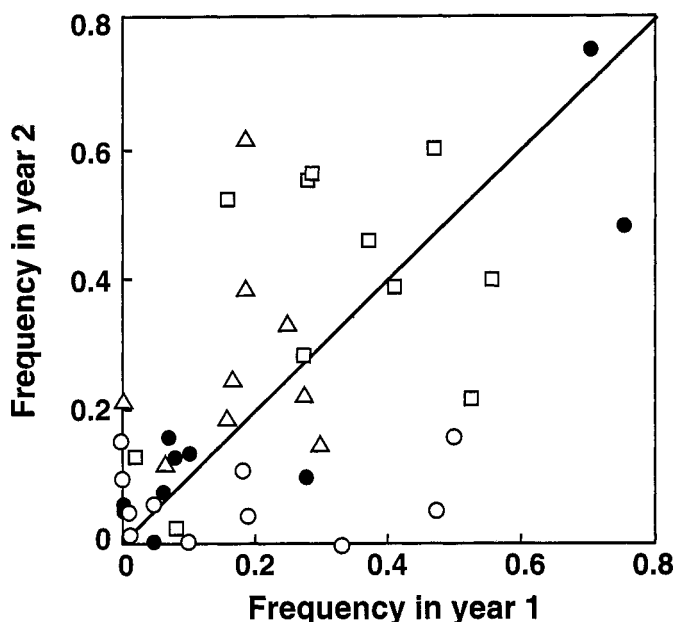


Fig. 14.5. Comparisons of year-to-year changes in the frequency of four pathotypes of *Melampsora lini* (● = A, ○ = E, □ = K and △ = N) occurring in nine populations. Frequency comparisons are variously for the years 1987–88, 1988–89 and 1989–90. Pathotypes absent in both years of a comparison are ignored.

population of *M. lini* present on *L. marginale* at Kiandra (Fig. 14.4). In that instance, the novel pathotypes were initially identified by their unique pathogenicity profile and confirmed as novel by isozyme analysis. From an initial combined detection frequency of 1% in 1988, these pathotypes rose in 2 years to comprise 34% of the population before abruptly disappearing completely (Burdon and Roberts, 1995).

Comparison of the pathotype structure of the pathogen populations at Kiandra and P1 also provides evidence of differentially applied, local, host-induced selection. Thus pathotype E is consistently present at site P1 where it is capable of attacking more than 85% of the host population. In contrast, it has never been recorded at the main Kiandra site where less than 2% of plants are susceptible to this pathotype.

Host population structure

To this point, I have concentrated exclusively on the dynamics and genetic structure of pathogen populations in a metapopulation framework. Data pertaining to temporal change in the resistance structure of host populations is

more limited, although much is known about spatial variability in the distribution of resistances among host populations.

At the broadest physical scale, patterns in the distribution of resistance are frequently associated with consistent environmental differences that differentially affect pathogen development (Dinoor, 1970; Burdon *et al.*, 1983). However, even within areas generally favourable for a pathogen, host populations often vary in both the number and frequency of resistant phenotypes they contain. Such variation may occur over distances as small as a few tens of metres (Parker, 1985). In the patchwork of *L. marginale* populations occurring in the Kiandra region of New South Wales, resistance to *M. lini* varies markedly between populations separated by only a few hundreds of metres (Jarosz and Burdon, 1991; J.J. Burdon, unpublished data). At its extreme this is demonstrated by differences between the Kiandra main site and site P1. At these two sites, the frequency of individuals carrying no detectable resistance varied between approximately 2 and 85% respectively, while the number of separate resistance genes or alleles varied between a minimum of seven and two (Jarosz and Burdon, 1991; Burdon, 1994).

In contrast to spatial variation, temporal changes in the frequency of resistance phenotypes in individual wild plant populations are very poorly documented. However, a detailed study of part of the *L. marginale* population occurring at the Kiandra main site shows the consequences for both host plant numbers and the genetic structure of the population, of an epidemic of *M. lini* (Burdon and Thompson, 1995). Following that epidemic, the numbers of *L. marginale* plants fell by 62% with heavily infected individuals suffering a disproportionately greater risk of death than lightly infected ones. This was accompanied by a marked change in the resistance structure of the population. The three resistance phenotypes that were most common before the epidemic showed a substantial decline in frequency, while other previously less significant phenotypes generally increased (Fig. 14.6). However, these changes in frequency showed no obvious adaptive value, as the structure of the pathogen population during the epidemic was dominated (54%) by a pathotype that was virulent on all the host phenotypes. Pathotypes in the remaining fraction of the pathogen population all showed differential ability to attack lines in the host population, but this was not reflected in increased survival of resistant lines. Indeed, there was a tendency for the more susceptible host phenotypes to increase in frequency (for example, phenotypes VI and IX, Fig. 14.6). Results of this kind are not particularly surprising given that plant breeding systems, through their effect on recombination and the development of linkage blocks (and *Linum* at this site is a tight inbreeder), may have a very marked effect on the outcome of selective episodes (Parker, 1991).

A similar non-adaptive response was detected in a population of the annual legume, *Amphicarpa bracteata*, attacked by *Synchytrium decipiens* (Parker, 1991). This raises a question about the function of resistance controlled by major genes in host populations. Initially, when a pathogen population

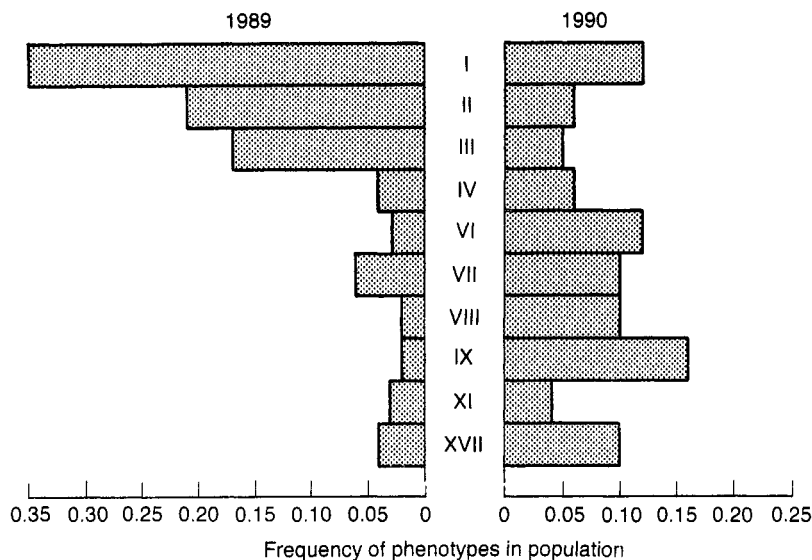


Fig. 14.6. Changes in the frequency of ten resistance phenotypes in a population of *Linum marginale* following a rust epidemic caused by *Melampsora lini* (data from Burdon and Thompson, 1995).

is composed of only one or two pathotypes (the original migrants into a previously pathogen-free host population), some host phenotypes may remain resistant and hence gain a selective advantage over others that are susceptible. However, when the pathogen population has been resident for a prolonged period, it may acquire by mutation, recombination and or migration, pathotypes that are locally capable of attacking all or most host resistance phenotypes. In these circumstances, periodic extinction of the entire pathogen population (as is envisaged under the metapopulation model) provides a means whereby the protection potentially afforded by all resistance genes is renewed (Burdon *et al.*, 1996). Following extinction, all incoming pathogen migrants will have to run the gauntlet of the 'renewed' resistances in the host population. The temporal respite this will provide for a host population is not fixed. Rather it will be determined by the immigration rate and the frequency of matching virulent pathotypes in the surrounding pool of pathogen populations.

Coevolution in the Longer Term

The raw material upon which selection, drift and stochastic forces act to generate the complex spatial and temporal patterns described above is clearly

genes or alleles for resistance in the host and pathogenicity in the pathogen. However, given the apparent imbalance built in to gene-for-gene interactions – new virulence being generated more easily than new resistance – a major question concerning the long-term dynamics of coevolving gene-for-gene systems focuses on the origin of these genes and alleles. Are they all derived from distant past events that occurred during the early establishment of particular associations or is their generation a continuing process?

In this regard the genetic control of resistance and virulence makes for very different levels of complexity and interest. For pathogen avirulence/virulence, the answer is relatively simple and the process has long been recognized as dynamic. With virulence generally being a simple loss of function and recessive to avirulence, the generation of new pathogen specificities can easily arise through simple deletion events or other inactivation mechanisms. Estimates of the rate of spontaneous mutation to virulence are few, but those available indicate considerable variation ranging from as high as 4.7×10^{-4} (Statler, 1990) and 1×10^{-5} (Flor, 1958) to less than 2×10^{-8} (Torp and Jensen, 1985). However, even at the lower end of the range, the high propagule production of individual lesions and the very large numbers of lesions occurring during disease epidemics ensure the frequent occurrence of mutant types. In most cases such changes will result in the 'single-step' increases in virulence that have been documented so often in detailed surveys of agricultural pathogens (Watson, 1981; Wellings and McIntosh, 1990). However, more dramatic changes are possible where avirulence genes occur in close proximity on the chromosome or where inhibitor genes exist that control the expression of pathogenicity at several avirulence loci simultaneously. Thus in *Melampsora lini*, a single dominant inhibitor gene has been shown to control the expression of five different avirulence loci simultaneously (Lawrence *et al.*, 1981; Jones, 1988).

On the plant side of the equation, resistance generally involves a positive gain of function and hence cannot be generated by simple mutation events (Pryor and Ellis, 1993). Despite this, large numbers of genes or alleles for resistance to a range of biotrophic pathogens have been detected in many agricultural (e.g. *Helianthus annuus*, *Hordeum vulgare* and *Triticum aestivum*) and wild (e.g. *Glycine canescens*, *Linum marginale* and *Senecio vulgaris*) plants. The frequent distribution of these resistances into allelic series or complexes of closely linked genes provides a clue as to their origin. Thus, for example 4, 2 and 5 linkage groups covering 13, 23 and 30 unique resistance specificities for resistance to *Bremia lactucae*, *Puccinia sorghi* and *Melampsora lini* have been found in *Lactuca sativa*, *Zea mays* and *Linum usitatissimum*, respectively (Hooker, 1985; Islam and Shepherd, 1991; Parin *et al.*, 1991). From these patterns Pryor (1987) suggested that novel specificities arise from a continuing process of unequal intragenic recombination or gene conversion events occurring during meiosis. Evidence for this idea has accumulated in a series of studies of the *Rp1* complex locus that codes for resistance to *Puccinia sorghi* in maize. In

that interaction, unstable events giving rise to new specificities caused by gene conversion (Hu and Hulbert, 1994) or unequal crossing-over (Pryor, 1987; Richter *et al.*, 1995) have been measured to occur with a frequency ranging between 6.7×10^{-3} and 1×10^{-2} (A.J. Pryor, Canberra, 1996, personal communication).

Detailed evidence of the type gathered for the *Rp1* locus takes considerable time and effort to accumulate, and is not available for wild host-pathogen associations. However, recently, substantially more linkage was found between seven resistance specificities found in a single population of *L. marginale* than between nine other host lines gathered from a diversity of sites (Burdon, 1994). Taking into account the breeding system of the host and the low isozymic diversity of the population in question, Burdon argued that the resistances may have arisen *de novo* on the site through a process similar to that proposed by Pryor (1987).

The possibility that new resistance specificities may evolve with frequencies in the range of 1×10^{-2} to 1×10^{-3} has considerable implications for our understanding of the long-term dynamics of gene-for-gene interactions. This would, at least partly, redress the frequently perceived imbalance between the rates of evolution of pathogenicity and resistance. Certainly the enormous population size differentials that are typical of host and pathogen populations do enhance the possibilities of changes in pathogenicity. However, the frequency with which allelic resistance series are encountered in gene-for-gene associations and the rate at which new resistance specificities may appear, suggests that these are still evolutionarily highly dynamic interactions in which factors like spatial isolation will be of great significance.

How Widespread are Gene-for-Gene Systems?

Gene-for-gene systems have received considerable attention in both experimental and theoretical studies of host-pathogen coevolution. From an agricultural point of view, where major gene resistance is widely used in breeding programmes, this emphasis is understandable. In contrast, although the presence of race-specific resistance is now well established in natural plant-pathogen associations (Burdon, 1987; Thompson and Burdon, 1992), the extent of its distribution and occurrence is still unclear. However, since the application of metapopulation thinking to host-pathogen coevolution, Burdon *et al.* (1996) have proposed that the development of gene-for-gene systems is likely to be favoured by situations in which substantial reductions in the size of pathogen populations occur and local extinctions of the pathogen are frequent. From this they argued that, with respect to the biology of pathogens, race-specific resistance genes should be commoner in associations in which the pathogen lacks an efficient means of off-season survival; while from the point of view of the plant, race-specific resistance genes should be more frequent in

annuals, or in perennial species in which the site of infection has an annual habit.

To date, the evidence available to test these ideas is limited. Certainly among agricultural crops, gene-for-gene systems are well represented by annual hosts (for example: *Avena*, *Glycine*, *Helianthus*, *Lactuca* and *Triticum*) or perennial hosts (*Malus* sp.) with annual infection targets (leaves). However, the occurrence of race-specific resistance genes in agriculture provides no measure of their distribution in natural systems (Barrett, 1985). Even excluding these associations, many of the essential elements of gene-for-gene systems have now been recognized in at least 15 natural host–pathogen interactions. These cover interactions between plants with a wide range of life forms – annual grasses and herbs, herbaceous perennials and even trees – and pathogens from a broad range of groups (*Ascomycetes*, *Basidiomycetes*, *Oomycetes*, *Fungi Imperfecti*) (Thompson and Burdon, 1992; Burdon *et al.*, 1996). A feature common to most of these interactions is the vulnerability of the pathogen population to massive and rapid reductions in population size as the supply of susceptible host tissue ceases. In contrast, pathogens that can survive in a quiescent state on dead material or invade perennial parts of the plant are far more likely to be part of a host–pathogen interaction in which resistance is quantitative in character (for example, *Phomopsis subordinaria* infecting *Plantago lanceolata*; De Noij and Van Damme, 1988) and may be based on a range of factors including morphological variation (for example, the *Ustilago violacea*/*Silene dioica* interaction; Elmqvist *et al.*, 1993).

Gene-for-gene systems are not the only coevolutionary interaction involving host resistance and pathogen pathogenicity/aggressiveness. However, they are a sound and logical place to start in developing a detailed understanding of the complexities of such interactions. Moreover, following the line of argument developed by Burdon *et al.* (1996), it seems likely that many more gene-for-gene systems will be uncovered as further studies are carried out and greater care is taken in distinguishing systems under polygenic control from gene-for-gene systems in which natural infection or mixed pathogen cultures generate patterns that superficially may seem best analysed by quantitative methods (Kinloch and Walkinshaw, 1991; Thompson, 1994).

Conclusions

The metapopulation view is currently a fashionable way of assessing a wide variety of population processes (Harrison, 1991; Hanski and Gilpin, 1991). However, while this approach has many attractive features for the study of host–pathogen interactions, not the least being its ability to make a virtue of the apparently stochastic nature of the spatial and temporal distribution of pathogens and disease epidemics, its relevance depends on the rate of extinction and migration in both host and pathogen. In essence, metapopulations lie

somewhere along a continuum of population distributions, ranging from situations where the exchange of genes between individual demes is sufficiently high as to prevent local differentiation, to situations where they are so widely separated that, for all intents and purposes, migration never occurs. A major challenge that lies ahead is to distinguish between these extremes, and to differentiate between metapopulations in which genetic drift and extinction are real possibilities in all populations, and situations in which the genetic and demographic dynamics of small peripheral populations are swamped by events occurring in adjacent large populations in which interactions are perennially played out (Harrison, 1991; Doak and Mills, 1994; Harrison and Hastings, 1996).

Achieving this requires the identification of potential but currently unoccupied population sites, long-term measures of extinction rates in individual populations of varying size and environmental vulnerability, and most difficultly, accurate measures of effective migration rates. As mentioned earlier, identification of migration events that result in the colonization of previously pathogen-free sites is relatively simple (Burdon *et al.*, 1995). However, these events only comprise a proportion of all those that actually occur. Migration that takes place when the target population already harbours the pathogen, may only be detected by subtle differences in pathogenicity or the presence of unique molecular markers (Watson, 1981; Burdon and Roberts, 1995). Moreover, even when the frequency of migrants entering a population is determined, its ultimate homogenizing effect on pathogen population structure across a series of demes will still be affected greatly by a diverse range of factors including the pathogen's breeding system, the relative fitness of migrants relative to incumbent pathogen isolates and the extent of temporal fluctuations.

Finally, in order to get a proper perspective of the place of gene-for-gene associations in the full spectrum of resistance mechanisms, we need to develop an understanding of the extent of their occurrence in natural systems and their interaction with more broadly based forms of resistance. Currently, our detailed knowledge of gene-for-gene systems in natural situations is limited to just two examples (*Linum marginale*/*Melampsora lini* and *Senecio vulgaris*/*Erysiphe fischeri*; Clarke *et al.*, 1990; Bevan *et al.*, 1993). Further studies involving: (i) other species combinations; (ii) the same associations under a range of very different environmental circumstances; or (iii) associations between a host and two or more pathogens differing in the genetic basis of their interaction with the host species; are now essential in order to explore the limits of gene-for-gene systems and the types of interaction in which they occur. As Thompson (1994) has pointed out, gene-for-gene interactions may well produce different evolutionary dynamics from more polygenic interactions between species. Through these approaches we should get a better understanding of the development of long-term interactions and the forces that determine the trajectory and pathways followed in coevolutionary interactions between plants and their pathogens.

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Cell Biology and Molecular Genetics



Genetics has transformed plant pathology on two occasions: at the turn of the century when Mendelian genetics enabled the discovery that disease resistance was a heritable trait in plants, and mid-century when H.H. Flor proposed the 'gene-for-gene' hypothesis to explain his observations of plant–parasite interactions. The transformation of plant pathology has continued with recent advances made possible by the application of recombinant DNA technology.

The most recent milestone emerged from progress in the past few years by several research groups using molecular genetic approaches to decode the first DNA sequences of plant genes required for disease resistance. In modern jargon, these naturally polymorphic determinants of genotype-specific disease resistance are called *R*-genes. Not so long ago, researchers would describe two possible scenarios in discussions about the molecular basis for this type of disease resistance in plants: resistance that was determined by a host gene which encoded a polypeptide capable of interacting directly with a product from the pathogen (produced by a so-called avirulence gene) and somehow causing resistance in a single step, or resistance that was conferred by a process of signal transduction in which the 'resistance' gene product serves as a pathogen perceptive molecule which in turn triggers a cascade of functionally conserved biochemical defence responses.

Given the enormous potential for adaptability in biological systems, one could have anticipated that both scenarios would prove to be valid, and in fact, examples of both have been revealed by molecular analyses. Tales of the unexpected have also been revealed such as dual specificity of a resistance gene, the possibility that the resistance gene product may be located in the cytoplasm instead of being membrane bound, and that a so-called *R*-gene may

not necessarily interact directly with an avirulence gene product of the pathogen. Genetic and molecular dissection of biochemical pathways in plants from pathogen perception to an effective defence response has defined a new frontier in plant pathology.

The impact of the recent molecular discoveries in rejuvenating debate is clearly evident in the research essays that follow. Molecular plant pathology is ultimately moving us towards an understanding of the physiology of disease resistance, and Mansfield *et al.* provide a summary of what is currently known about biochemical events which closely correlate with the phenomenon of disease resistance in several pathosystems. The tremendous variety of pathogen determinants of virulence are presented in chapters by Vivian *et al.*, Knogge and Marie, and Spence. They present recent progress in the understanding of avirulence in bacteria, fungi and viruses, respectively. A synthesis of what is currently known about host determinants of disease resistance is presented in the chapter by Beynon, and complements what has been presented in Part I in chapters written by Holub, Hulbert and Schultze-Lefert *et al.* Keen was invited to share his perspective in a chapter based on the Garrett Memorial Lecture he presented at the 1995 BSPP Presidential meeting. He was honoured for his central role in reformulating Flor's hypothesis for the modern age of molecular biology. The chapter by Dangl examines what potentially can be gleaned from the mammalian immune system to advance further our understanding of disease resistance in plants. Briggs and Kemble provide an industry perspective in the final chapter on the possible impact that recent discoveries will have in agriculture.

A clear message from all of these authors is that fundamental investigations of disease resistance in plants extend beyond the practical applications for crop improvement. At the very least, Flor's hypothesis offers a useful paradigm for research on disease resistance in animals. However, molecular plant pathology also provides even more as a powerful model for understanding environmentally induced signal transduction in general and the evolution of disease resistance in eukaryotes.

E.B. Holub

Phenotypic Expression of Gene-for-Gene Interaction Involving Fungal and Bacterial Pathogens: Variation from Recognition to Response

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It was apparent from the very earliest studies that varietal resistance may be expressed at different stages of the interaction between plant and pathogen, and that phenotypic variation reflects the presence of different resistance genes in the host plant. The first detailed histological studies of resistance were by Marryat (1907) who examined the infection of wheat by the yellow rust fungus, *Puccinia glumarum* (syn *P. striiformis*). In experiments comparing the susceptible variety Michigan Bronze with two resistant wheats, she clearly described the occurrence of a rapid hypersensitive reaction in Einkorn in which, '... the rapid breakdown and death of the host tissue in the parts attacked involves the death of the parasite'. Resistance was found to occur at a later stage of colonization in American Club in which, '... one is more conscious of a continuous struggle'. Her drawings illustrated observations made earlier by Marshall Ward (1902) in one of the classic studies of plant/microbe interactions which dealt with infection of *Bromus* species by brown rust *P. dispersa* (syn *P. recondita*). Ward found that fungal growth was restricted soon after stomatal penetration in some species, whereas in others some mycelial development was observed with weak or very poor sporulation.

Stakman (1915) studying resistance to *P. graminis*, first used the term 'hypersensitive' to describe, '... the abnormally rapid death of host plant cells when attacked by rust hyphae'. The description of infection types subsequently developed by Stakman and co-workers from their analysis of stem rust isolates and wheat cultivars provided further indication that each gene for resistance conferred a particular type of plant response. Infection development was scored on a 0 to 4 scale (no infection, to production of large uredia) as outlined

in Table 15.1. Stakman (1915) commented that the more resistant a plant, '... the quicker are a few host cells in the immediate neighbourhood of the invading hyphae killed and the sooner does the fungus itself cease activity'. He also pointed out that hyphae of the stem rust appeared to be actively inhibited within resistant plants and not simply to cease growth by starvation.

As will become apparent from subsequent discussion, we now have detailed knowledge of the structure of genes for avirulence (*A*) and resistance (*R*) and the gene-for-gene concept has been extended to cover both obligate and facultative parasites with quite different modes of infection development (Mansfield, 1990). Nevertheless, the early and perceptive work of Stakman and colleagues in studies of the biotrophic rusts raised the following questions which remain equally valid today.

1. Is resistance in gene-for-gene interactions always associated with plant cell death and the hypersensitive response (HR)?
2. Does the plant's resistance response occur only in cells in contact with the invading pathogen?
3. What is the cause of cell death?
4. What is the relationship between the timing of plant cell death and restriction of the pathogen?
5. Is cell death alone sufficient to account for the restriction of obligate parasites?
6. At what stage of infection are the signals which determine the outcome of the gene-for-gene interaction exchanged?

Table 15.1. Description of infection types used in classifying the reactions to stem rust on seedling wheat leaves (after Stakman *et al.*, 1962 and Knott, 1989).

Class	Infection type	Gene for resistance	Description of symptoms
Immune	0	<i>Sr5</i>	No signs of infection to the naked eye but minute flecks may be visible under low magnification
Very resistant	0;	<i>Sr6</i>	No uredia, but distinct flecks of varying sizes, usually a chlorotic yellow but occasionally necrotic
Resistant	1	<i>Sr11</i>	Small uredia surrounded by yellow chlorotic or necrotic areas
Moderately resistant	2	<i>Sr13</i>	Small to medium-sized uredia, typically in a dark green island surrounded by a chlorotic area
Moderately susceptible	3		Medium-sized uredia, usually surrounded by a light green chlorosis
Susceptible	4		Large uredia with a limited amount of chlorosis: may be diamond-shaped

7. Once activated, does the HR involve the same biochemical changes irrespective of the *R* and *A* gene combination that controls recognition?

With these questions in mind and in the context of phenotypic variation, we will base this article on experiments completed at Wye with gene-for-gene interactions between lettuce (*Lactuca sativa*) and *Bremia lactucae*, the cause of downy mildew disease. Comparisons will be drawn with other host/pathogen combinations including both fungal and bacterial pathogens.

Lettuce Downy Mildew

The genetics of resistance to *B. lactucae* is reviewed by Crute (1992). It was apparent from studies of Maclean and Tommerup (1979) that resistance governed by the *Dm* genes is often expressed by the HR of epidermal cells as observed by their cellular collapse and browning. One of the most controversial aspects of studies of gene-for-gene interactions has concerned the detection of cell death in cells undergoing the HR (Mansfield, 1986). We have used failure to plasmolyse as the parameter to identify dead cells in lettuce cotyledons. This method has the advantage that it is a physiological probe which can be applied at the cellular level; plasmolytic failure indicates the occurrence of irreversible damage to the plasma membrane. There is no doubt that cells with irreversible membrane damage (IMD) are unable to recover, but many biochemical changes, particularly those involving oxidative processes, can occur in cells after IMD and subsequent decompartmentation and organelle disruption. Most notably, in lettuce, cells do not become discoloured until many hours after IMD. In short, a dead (necrotic) cell is not necessarily a brown cell! The processes involved in necrobiosis have recently attracted considerable attention because of the possible analogy between the HR and programmed cell death, particularly apoptosis, in animal cells (Sen, 1992; Collins and Rivas, 1993; Dietrich *et al.*, 1994; Greenberg *et al.*, 1994; Levine *et al.* 1994; Mittler *et al.*, 1995; Jones and Dangl, 1996; Ryerson and Heath, 1996).

In our experiments, we have used a range of isolates with defined genotypes including the isolate VO/11 which is known to be heterozygous at ten avirulence loci (kindly provided by Pam Gordon and Ian Crute, HRI, Wellesbourne). By recording the characteristic fungal structures produced and the ability of penetrated lettuce cells to plasmolyse, it is possible to determine the stage of fungal development at which IMD typically occurs for any *Dm* gene interaction. Figure 15.1 summarizes the stages at which IMD was found to occur. In those reactions which might be considered immune, for example with cvs Blondine (*Dm1*), UCDM2 (*Dm2*), Dandie (*Dm3*) and Valmaine (*Dm5/8*), a rapid HR was observed in epidermal cells but differences in the timing of IMD were revealed. For example, expression of *Dm5/8* resulted in IMD during the expansion of the primary vesicle within 1.5 h of penetration, whereas IMD was

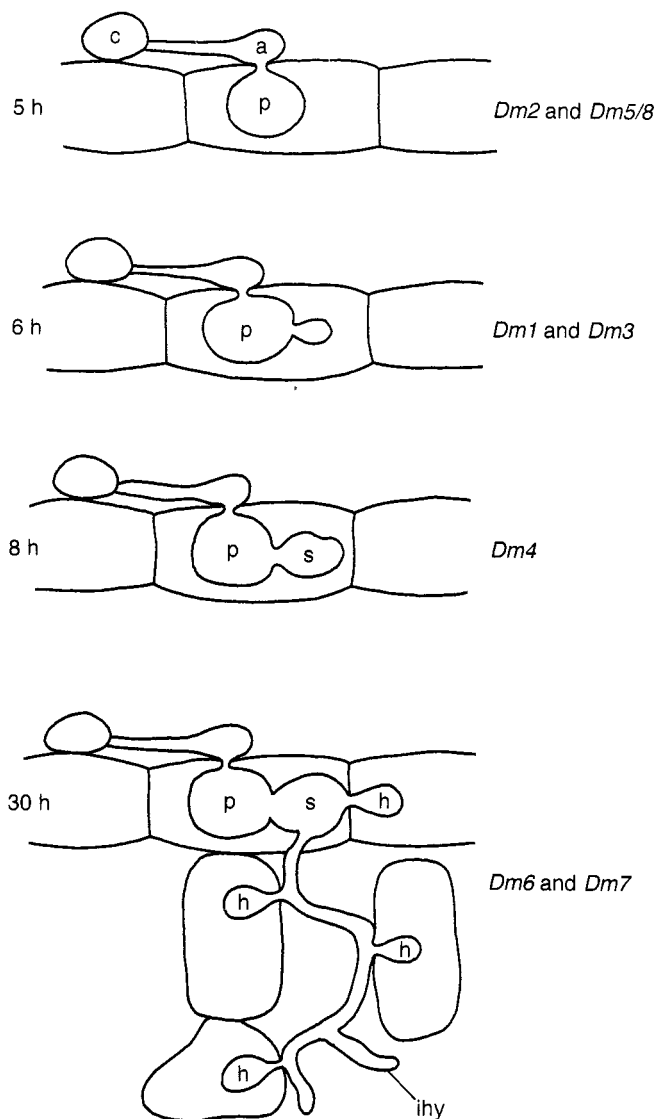


Fig. 15.1. Timing of irreversible membrane damage (IMD) determined by genes for resistance to downy mildew in lettuce. Progress of infection by *Bremia lactucae* and the stages and times after inoculation at which IMD occurs in penetrated cells are illustrated. Fungal structures labelled are: c, conidiosporangium; a, appressorium; p, primary vesicle; s, secondary vesicle; h, haustorium and ihy, intercellular hypha.

delayed with *Dm3* until the secondary vesicle was well established. With genes conferring intermediate resistance, for example *Dm6* and *Dm7*, which may allow some weak sporulation, no IMD occurred in epidermal cells during the first 12 h after inoculation (Woods *et al.*, 1988) but mesophyll cells died shortly after penetration by haustoria. Despite the different timing of responses, all resistance genes led to the HR only in cells penetrated by the fungus, and plant cell death (as determined by IMD) clearly preceded restriction of fungal growth.

Although isogenic lines differing only in the presence or absence of *Dm* genes were not available, comparison of the phenotypes observed in different cultivars carrying the same *Dm* gene indicated little effect of genetic background on the interaction. It was clear that the more rapidly acting forms of resistance were epistatic. However, slight variations were noted between cultivars; for example the greater tendency of cells of cv. Diana penetrated by V0/11 to undergo IMD during the early stage of primary vesicle expansion, before it was observed in cv. Valmaine (also *Dm5/8*). In all cases, the occurrence of IMD clearly preceded the appearance of cytoplasmic collapse and cellular browning characteristic of the later stages of the HR.

Variations between the *Dm* genes were not only confined to the timing of IMD. Striking differences were found in the responses to heat-shock of cultivars with *Dm5/8* and other *Dm* genes (Woods *et al.*, 1989; Woods-Tör *et al.*, 1991). Treatment at 55°C for 45 s rendered cotyledons of cv. Valmaine (*Dm5/8*) susceptible to isolate V0/11; no HR developed for several days and some sporulation was observed. In the untreated tissue expressing *Dm5/8*, fungal growth ceased during the first day after inoculation (see Fig. 15.1). The effects of heat-shock were temporary with other *Dm* genes; occurrence of IMD was delayed but not for more than 17 h (Woods *et al.*, 1989). The specific effect of heat-shock on *Dm5/8* was suggested to involve effects on early perception of elicitors perhaps involving the *Dm* gene product itself. The more transient effects on the expression of resistance conferred by other genes (i.e. *Dm1*, *Dm3* or *Dm7*) was attributed to temporary suppression of protein synthesis controlling IMD and other defence responses.

As predicted from the classical concept of the HR, cytoplasmic collapse would be expected to cause starvation of the obligate parasite *B. lactucae*. In support of this hypothesis, it is notable that production of secondary vesicles is almost immediately disrupted following IMD in epidermal cells expressing the rapid HR such as *Dm3* or *Dm5/8* (Fig. 15.1; Woods *et al.*, 1988). The secondary vesicle, instead of expanding to fill the cell, often develops into an intracellular hypha and rapidly grows out of the epidermis. Such a rapid change in morphology suggests disruption of a nutrient source or release of some fungitoxic principal.

Although plant cell death *per se* may contribute to restriction of colonization by *B. lactucae*, the lettuce must have other defence responses to prevent invasion by facultative parasites such as *Botrytis cinerea*. Two mechanisms of resistance, phytoalexin accumulation and the deposition of phenolics in cell

walls, have now been identified in lettuce tissues challenged by *B. lactucae*. Lettucenin A is the major phytoalexin and its accumulation has been closely associated with necrosis, the yields of lettuценin A being directly related to the numbers of cells undergoing the HR in challenged tissue (Bennett *et al.*, 1994).

Lettucenin A fluoresces under UV radiation but attempts to use fluorescence to determine the cellular localization of the phytoalexin have been hampered by the deposition of autofluorescent phenolics in responding cells (Bennett *et al.*, 1994, 1996). The advantage of recent studies on phenolic deposition has been that, like IMD, the response can be examined within whole cotyledons and defined at the subcellular level. Using the two approaches has allowed the timing of deposition of autofluorescent phenolics to be studied in relation to the occurrence of IMD during gene-for-gene interactions. Figure 15.2 illustrates the autofluorescence observed in cells undergoing the HR. Following penetration, autofluorescent deposits were first observed in the cell wall around the penetration point. Fluorescence then spread into the surrounding wall and intracellular fungal structures. In lettuce, induced autofluorescence is not associated with widespread lignification; mild alkaline hydrolysis removes fluorescent material, releasing caffeic acid and syringaldehyde (Bennett *et al.*, 1996).

The relationship between IMD and deposition of phenolics found in epidermal cells of cv. Diana challenged by isolates VO/11 causing a rapid HR

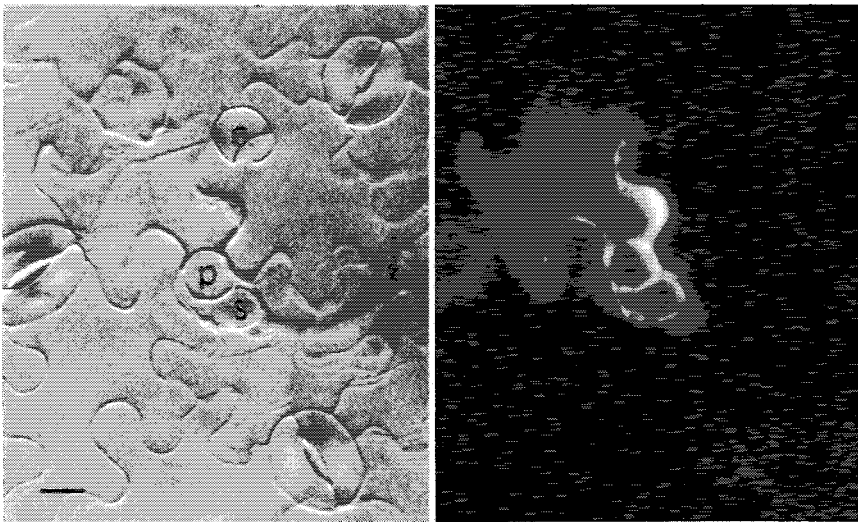


Fig. 15.2. Autofluorescence in an epidermal of lettuce cv. Diana undergoing the HR after penetration by *Bremia lactucae* isolate CL9W. Tissues were cleared in methanol and chloral hydrate before examination under tungsten illumination with differential interference contrast (a) or blue light excitation (b). Bar = 20 μ m; c, conidiosporangium; p, primary vesicle; s, secondary vesicle; arrow, intercellular hypha. (Adapted from Bennett *et al.*, 1996.)

(*Dm5/8*), CL9W causing a later HR (*Dm7*), and TV, a virulent strain (with no matching avirulence genes) is summarized in Fig. 15.3. The quantitative analyses clearly emphasize the differing timing of IMD in the *Dm5/8* and *Dm7* interactions and also differences in phenolic deposition. The spread of autofluorescence before IMD was much greater in the *Dm7* interaction as indicated by the high percentage of plasmolysed (live) cells with category 3 fluorescence. With V0/11 and *Dm5/8*, IMD was often observed *before* any deposition of autofluorescent phenolics. In each interaction, widespread fluorescence (categories 4 and 5) was observed only in cells which failed to plasmolyse. The major response, therefore, followed epidermal cell death and may in part be linked to the synthesis of phenolics in surrounding cells (Bennett *et al.*, 1996).

The occurrence of IMD during the HR determined by *Dm5/8* and *Dm3* has been shown to be delayed by treatments with Blastidicin S (BcS), an inhibitor of protein synthesis but not by cordycepin or actinomycin D, which affect mRNA synthesis (Woods *et al.*, 1989). The effects of BcS and cordycepin and also amino-oxycacetate (AOA), a competitive inhibitor of phenylalanine ammonia lyase (PAL, Amrhein *et al.*, 1976), on the deposition of autofluorescent phenolics was examined during the rapid HR in cv. Valmaine (*Dm5/8*) challenged by V0/11. Following treatment, the spread and intensity of fluorescence was examined in cleared cotyledons 24 h after inoculation. Although only BcS delayed the occurrence of IMD (Woods *et al.*, 1989 and unpublished data), all treatments reduced the autofluorescence response. Particularly striking was the overall reduction in intensity of fluorescence after treatment with each of the inhibitors and the greatly reduced spread of fluorescence away from the penetration point in tissues treated with Blastidicin S or cordycepin but not AOA. In conclusion, localized deposition of some phenolics around the penetration point appeared to be unaffected by inhibitors, but the spread and accumulation of fluorescing material required both mRNA and protein synthesis, and PAL activity. Each of the inhibitors allowed the production of more intercellular hyphae at infection sites than observed in controls treated with water alone. Growth was, however, still greatly restricted in comparison with that observed in the susceptible cv. Cobham Green.

Despite the change in fungal development associated with IMD in lettuce, it appears that cell death does not alone cause the observed inhibition of *B. lactucae*. This is because treatments with actinomycin D, cordycepin or AOA do not prevent IMD but do allow some further fungal growth. The implication here is that defence products such as phenolics and phytoalexins contribute significantly to inhibition of the obligate parasite. It also seems that, although similar biochemical changes may be activated, the rate at which responses occur within the penetrated cell may vary depending on the *Dm* gene. Thus *Dm5/8* controls rapid changes in membrane permeability whereas *Dm7* is characterized by a more gradual loss of membrane function, allowing greater metabolic activity *before* IMD.

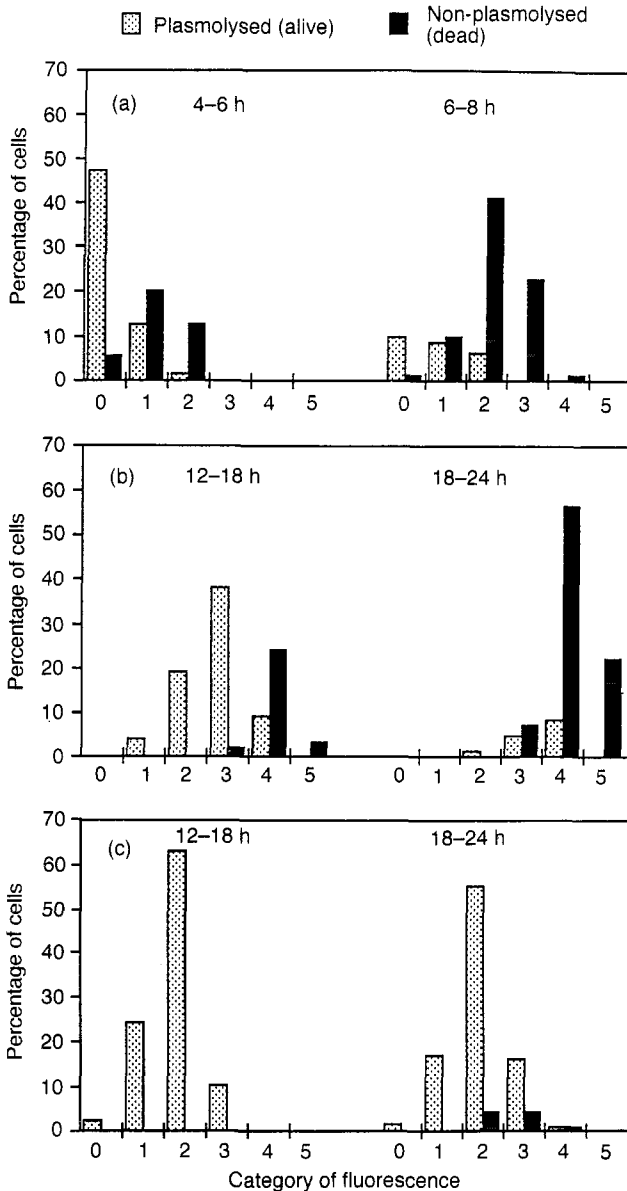


Fig. 15.3. Relationship between the occurrence of irreversible membrane damage and the appearance of yellow autofluorescence in epidermal cells of lettuce cv. Diana penetrated by isolates (a) V0/11, (b) CL9W (both incompatible) and (c) TV (compatible interaction). At least 50 conidiosporangia were examined in each time period. Note the much earlier response to V0/11 during expression of *Dm5/8*. Spread of fluorescence was scored as follows: 0, none; 1, around penetration point; 2, as 1 and extending into the surrounding wall; 3, as 2 with more spread of fluorescence into intracellular fungal structures; 4, fluorescence widespread throughout the walls of the penetrated cell; 5, as 4 with aggregates of brightly fluorescing material as shown in Fig. 15.2. (Adapted from Bennett *et al.*, 1996.)

The cause of cell death remains unknown, but the requirement for protein synthesis in the penetrated cell, as indicated by BcS and heat-shock sensitivity, suggests that there is a form of programmed cell death in action. Although treatment with actinomycin and cordycepin did not inhibit all mRNA synthesis in treated cotyledons (Bennett *et al.*, 1996), the implication from the differential effects observed with inhibitors is that the rapid IMD in cultivars with *Dm5/8* is not regulated at the level of transcription, whereas the major secondary response of phenolic synthesis and deposition does require new transcripts. It is clear that deposition of autofluorescent phenolics does not itself cause IMD with either *Dm5/8* or *Dm7*.

Despite the biochemical characterization of responses occurring at the cellular level in lettuce downy mildew, we have no clues concerning the nature of the initial signalling event. Although there have been numerous attempts to recover gene-specific elicitors of the HR from infection structures of *B. lactucae* and intercellular washing fluids from infected tissue, none has been successful (Crucefix *et al.*, 1987; Mansfield *et al.*, 1988). Because the timing of IMD appears so closely related to development of intracellular structures (e.g. with *Dm1*, *Dm3* and *Dm5/8*), it seems probable that expression of the matching *A* gene is associated with developmental changes leading to altered morphology such as the transition from primary to secondary vesicle, and secondary vesicle to intercellular hypha (Fig. 15.1). The direct contact between intracellular structures and the plant cell membrane (as occurs with all obligate parasites forming haustoria) allows the activity of the widest possible range of signalling molecules, including components which might not be released from the surface of the fungal wall. Until the elicitors and receptors have been identified, our knowledge of the links between recognition, signal transduction and the defence response in lettuce controlled by different *Dm* genes must remain speculative. Of particular interest in this interaction is the speed at which IMD occurs during expression of *Dm2* and *Dm5/8*. Several pieces of the gene-for-gene jigsaw have been studied in detail, but it will not be possible to complete the picture without the identification of the ligand–receptor complex.

Barley Powdery Mildew

Barley powdery mildew disease caused by *Erysiphe graminis* f. sp. *hordei* provides a useful comparison with lettuce downy mildew. Like *B. lactucae*, *E. graminis* is an obligate parasite which penetrates directly into epidermal cells. Infection development is summarized in Fig. 15.4. Intercellular mycelium is not produced, but the fungus grows on the leaf surface producing haustoria in underlying epidermal cells. Within 3 to 4 days after inoculation, a fine web of mycelium can be seen as a young mildew colony on the leaf surface of susceptible cultivars. The colony subsequently produces visible mats of mycelium and conidial chains in their thousands. The phenotype of the interaction is usually

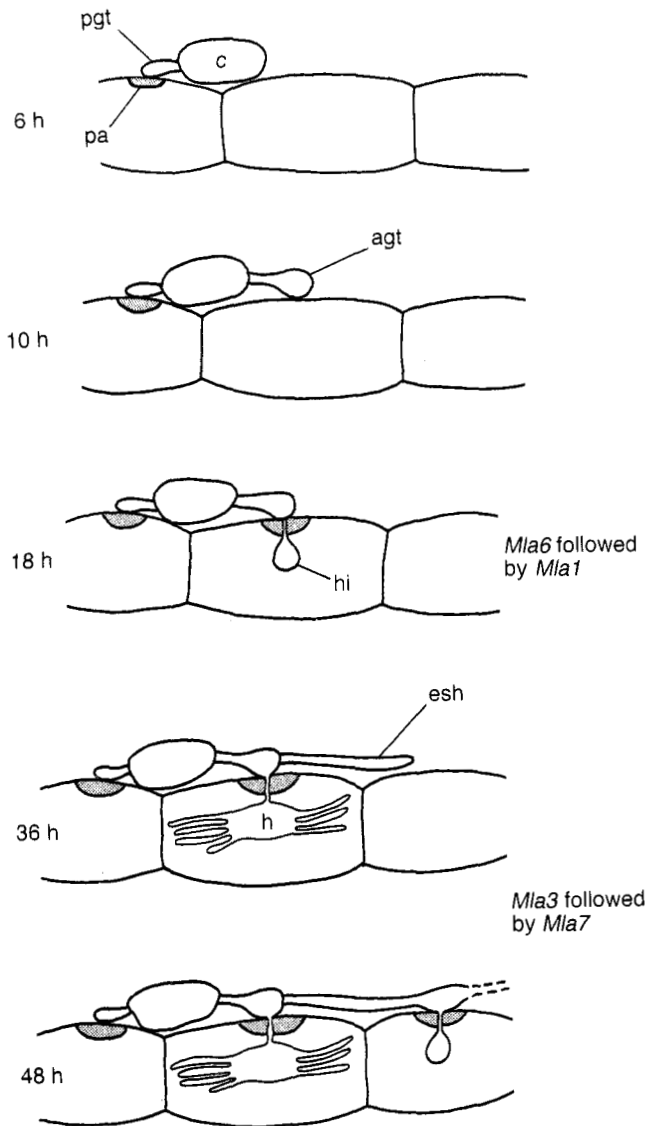


Fig. 15.4. Timing of cytoplasmic collapse determined by genes for resistance to powdery mildew (*Ml*) in barley. Progress of infection by *Erysiphe graminis* and the stages and times after inoculation at which responses (including irreversible membrane damage) occur are illustrated. Fungal structures labelled are: c, conidium; pgt, primary germ-tube; agt, appressorial germ-tube; hi, haustorial initial; h, mature haustorium; esh, elongating secondary hypha. The papilla reaction (pa) within challenged cells is also marked. Note that the primary germ-tube induces papilla deposition but does not penetrate through the epidermal cell.

recorded when colonies have started to produce conidia on the fully susceptible cultivars, an interaction classified as infection type (IT) 4. Resistant cultivars which develop no easily visible symptoms are ascribed IT0; some brown flecking and limited mycelial development, IT1; flecking necrosis and chlorosis with some sporulation, IT2; chlorosis but little flecking necrosis and only small sporulating colonies produced, IT3 (Moseman *et al.*, 1965; Moseman, 1972). The genetics of powdery mildew resistance in barley was recently reviewed by Jørgensen (1994).

There have been numerous studies of infection development (see reviews by Carver, 1988 and Aist and Bushnell, 1991). Two defence responses have been described: the prevention of penetration by the formation of a papilla and the hypersensitive collapse of cells following initiation of the haustorium (Koga *et al.*, 1988). The papilla response is particularly important in *mlo*-determined race non-specific resistance which is not based on a gene-for-gene interaction (Freialdenhoven *et al.*, 1996; see also Schulze-Lefert *et al.*, Chapter 3 this volume). There are many genes for race-specific resistance to powdery mildew and, of these, 28 alleles map to the *Mla* locus on the short arm of chromosome 5, which also carries *Mlat*, *MIga*, *MIk*, *MIln* and *MIra* (Jørgensen, 1994). Within the *Mla* locus, different alleles show characteristic macroscopic infection types ranging from fully susceptible to immune.

Recent experiments by Boyd *et al.* (1995) used isogenic lines of cv. Pallas containing one of four *Mla* genes which conditioned different ITs, i.e. *Mla1* (IT0), *Mla6* (IT0), *Mla3* (IT1–2) and *Mla7* (IT2–3), to isolates of *E. graminis* containing matching avirulence genes. The occurrence of the HR in challenged tissues was assessed by the accumulation of autofluorescent phenolics throughout the responding cell. The pattern of infection development and occurrence of the HR (Fig. 15.4) was similar to that we have observed in *Bremia*/lettuce interaction. The low ITs were associated with rapid HR in epidermal cells followed by early restriction of growth after the formation of rudimentary haustoria. Fungal growth, penetration and cell collapse were, however, inherently more rapid in the lettuce system. The low ITs observed in barley with *Mla1* and *Mla6* were also associated with a slightly higher frequency of papilla formation. Where colonies developed (ITs 2 and 3), the HR followed the establishment of mature haustoria within epidermal cells and extended from penetrated cells to underlying unpenetrated mesophyll tissues, leading to the development of a macroscopic fleck.

The extension of necrosis into tissue surrounding the penetrated cell is also a feature of the expression of certain genes for rust resistance in the cereals (see Table 15.1) and the reaction of *Arabidopsis* to *Peronospora parasitica* (Holub *et al.*, 1994). In all cases, necrosis appears to progress from the central focus of the penetrated cell. Whether or not the same elicitors are involved in triggering cell death in penetrated and surrounding cells is not known. Phenotypes with the more extensive necrosis are always associated with greater fungal growth than observed in reactions in which only the penetrated cell undergoes the HR.

suggesting that in the former case, a delayed but eventually greater production of a diffusible elicitor may be the simple answer.

Boyd *et al.* (1995) attempted to correlate ITs with appearance of transcripts for defence-related genes. Their analysis did not reveal striking differences between Pallas and inbred lines with low ITs, but significant increases in chitinase, peroxidase and PR-1 transcripts were seen in *Mla3* and *Mla7* lines late in the interaction, associated with the more widespread HR observed in these genotypes. There is a need for *in situ* hybridization experiments to analyse responses at the cellular level in barley, as pioneered by Schmelzer *et al.* (1989) in their studies of resistance in parsley. The more precise temporal and spatial analysis afforded by *in situ* studies may allow a differential pattern of gene expression within penetrated barley cells to emerge.

There are fundamental differences between the *Bremia*/lettuce and *Erysiphe*/barley systems. Papilla formation is not a component of gene-for-gene interactions in lettuce and only penetrated cells appear to undergo the HR in the *Dm/A* interactions examined. Further differences are apparent from studies on the effects of inhibitors on autofluorescence and cell death. In contrast to the lettuce/*Bremia* interaction, cordycepin, PAL and cinnamyl alcohol dehydrogenase (CAD) inhibitors were found to prevent not only the accumulation of phenolics but also plant cell death in barley (Zeyen *et al.*, 1995). The proposal that the accumulation of phenolics within responding cells may be the cause of the HR in cereals was suggested by Moersbacher *et al.* (1990) working with the *Sr5* gene for stem rust resistance in wheat. Such a relationship may also apply in barley leaves, but coleoptile tissue cell death during the HR is not associated with such a strong accumulation of autofluorescence. Zeyen *et al.* (1995) argued that the effects of the CAD inhibitor on cell death were not due to phenolic accumulation *per se* but to certain products of CAD activity (such as lignin precursors) being, '... necessary in the chain of events leading to programmed cell death conditioned by *Mla1*'. They proposed that, '... lignin precursors may stimulate peroxidase mediated synthesis of H₂O₂ which may be part of ... the cell death phenomenon'.

Tomato Leaf Mould

The interaction between tomato and *C. fulvum*, the cause of leaf mould, has become one of the most well characterized gene-for-gene systems. Both components, resistance genes in the plant and avirulence genes in the pathogen, have been cloned and sequenced. The elicitors of the plant's defence responses have been identified to be processed protein products of avirulence genes (Schottens-Toma and de Wit, 1988; Van den Ackerveken *et al.*, 1993; Jones *et al.*, 1994; Joosten *et al.*, 1994; de Wit, 1995; Hammond-Kosack and Jones, 1995; Dixon *et al.*, 1996). Unfortunately, the tomato leaf mould fungus is not a good model as a biotrophic fungal pathogen. Unlike the rusts and mildews,

which have formed the basis for development of the gene-for-gene concept, *C. fulvum* does not penetrate into plant cells or produce haustoria. Growth of virulent isolates progresses through the intercellular spaces without causing obvious damage to adjacent plant cells, until sporulation occurs through stomatal pores, about 10 days after inoculation. In resistant plants, isolates penetrate stomata to enter the leaf, but their growth is restricted and they fail to sporulate (de Wit, 1977). The extent of fungal growth is dependent on the avirulence (*avr*) and resistance (*Cf*) gene combination.

Hammond-Kosack and Jones (1994) described a detailed study of the expression of resistance conferred by the *Cf-avr* gene combinations. Measurements of fungal growth revealed that the relative efficiencies of the *Cf* genes decreased in the following order (days after inoculation when no fungal growth was observed are given in parentheses): *Cf-2* (6.4), *Cf-5* (8.1), *Cf-9* (9.3), *Cf-4* (12.9) and *Cf-3* (16.2). The phenomenon of gene dosage, another source of phenotypic variation which has also been described for the *Dm6* gene for resistance to lettuce downy mildew (Crute, 1992), several alleles of the *Mla* locus in barley (Jahoor *et al.*, 1993) and genes for resistance to downy mildew in *Arabidopsis* (Holub *et al.*, 1994; Holub and Beynon, 1996), was examined in detail. Plants homozygous for each of the *Cf* genes were more effective in containing infection than their heterozygous counterparts. The great advantage of the *C. fulvum* system is that gene-specific elicitors of responses accumulate in intercellular fluids which can be recovered from compatible interactions, such as race 0 (with all *avr* genes) growing on a cultivar without *Cf* genes for resistance (de Wit and Spikman, 1982). Using such a preparation of elicitors, Hammond-Kosack and Jones (1994) showed that the homozygous plants also responded to a twofold lower concentration of the elicitor.

The effects of elicitors from intercellular fluids are to cause yellowing, discoloration and eventually necrotic collapse of infiltrated tissue. In early studies, these reactions were considered to represent induction of the HR, but it is now clear that the resistant reactions of tomato to *C. fulvum* are typically not associated with the rapid onset of plant cell death as observed with *Bremia* or *Erysiphe*. Necrosis in tomato, as indicated by IMD and loss of compartmentation in response to avirulent isolates of *C. fulvum* or elicitors, usually involves a prolonged period of cellular activity. Hammond-Kosack and Jones (1994) considered that the *Cf-2*-mediated reactions most closely paralleled a classical HR because stomatal guard cells often collapsed following contact with avirulent *C. fulvum* hyphae. However, Lazarovits and Higgins (1976) had earlier pointed out from studies of cv. Vinequeen (expressing *Cf-2* + *Cf-4*) that underlying mesophyll cells in contact with hyphae often became rounded and showed cell wall thickening (callose deposition) but not collapse typical of the HR. Large deposits of phenolic material were also observed within the intercellular spaces often in contact with distorted hyphae. In other *Cf* gene interactions, no macroscopic symptoms are observed in epidermal cells. Cell swelling and wall alterations appear at progressively later stages of the interaction, timing of the

response being directly correlated with the restriction of fungal growth. Inhibition of *C. fulvum* appears to be due to the localized generation of an antimicrobial environment caused by a combination of phenolics, the deposition of callose and phytoalexin accumulation (Lazarovits and Higgins, 1976; de Wit and Flach, 1979). A possibly direct, antifungal role for active oxygen species (AOS) generated by the oxidative burst, which is an early response to the elicitors, has also been suggested (Vera-Estrella *et al.*, 1992; Hammond-Kosack and Jones, 1994).

The availability of gene-specific elicitors allowed Hammond-Kosack *et al.* (1996) and May *et al.* (1996) to examine the sequence of biochemical and physiological changes occurring within tomato leaves undergoing Avr2/Cf-2- and Avr9/Cf-9-mediated reactions. Use of elicitors ensures that most cells will be responding and avoids the difficulties associated with highly localized responses occurring within tissues challenged by isolated fungal hyphae. The reactions observed and their timing following exposure of cells to elicitor preparations are summarized in Fig. 15.5. In the context of the HR and cell death, what is immediately apparent is that loss of cell viability (measured by methods based on plasma membrane integrity) occurs at different times depending on the Cf and avr gene interaction and is by no means an early response. An intriguing feature of this analysis is that, in the plant, the Cf-2-based resistance is expressed much more rapidly than Cf-9, whereas the opposite is found when

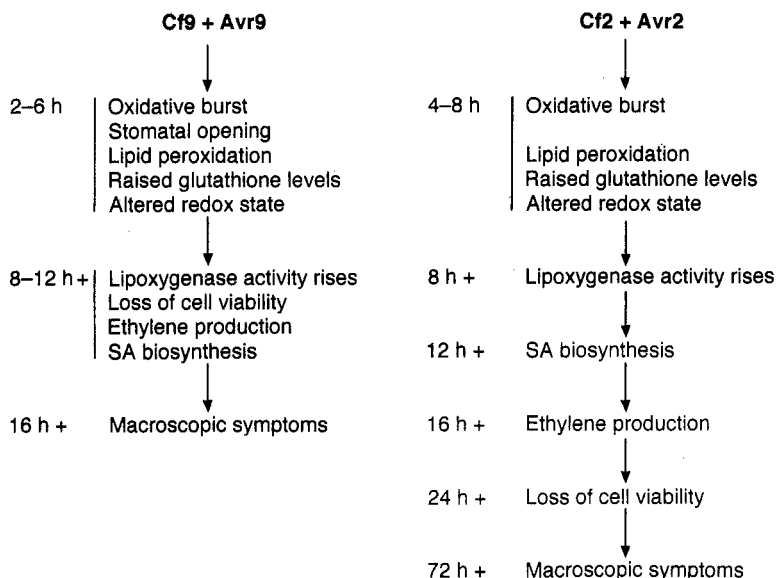


Fig. 15.5. Sequence of responses in leaves of tomato cultivars with the Cf-2 or Cf-9 genes following injection with intercellular fluids containing race-specific elicitors derived from *avr2* and *avr9*. (Adapted from Hammond-Kosack *et al.*, 1996.)

working with elicitors. The apparent contradiction between responses to the elicitors and the fungus may be linked to the timing of expression of the *avr2* and *avr9* genes within the infected plant. It is known that *avr4* and *avr9* are not induced by the same conditions *in vitro* (Van den Ackerveken *et al.*, 1993; Joosten *et al.*, 1994; de Wit, 1995). Differential timing of *avr* gene expression as well as differential effects of elicitors on plant responses are therefore implicated in the varied phenotypes associated with resistance to *C. fulvum*.

Resistance to Bacteria

There are numerous examples of gene-for-gene interactions between bacterial pathogens and their plant hosts. Vivian *et al.* (Chapter 16 this volume) describes features of 50 alleles for avirulence which have been cloned from species of *Pseudomonas* and *Xanthomonas*. All of the bacteria occupy the same niche within the plant, multiplying within the intercellular spaces as described for *Cladosporium fulvum*. Resistance is typically associated with hypersensitivity (Klement, 1982; Mansfield and Brown, 1986). As observed with fungal pathogens, there are numerous examples of variation in the macroscopic symptoms associated with resistance, reflecting the timing of the onset of cell collapse during the HR. One of the more striking differences in timing occurs with the *Bs1*, *Bs2* and *Bs3* genes for resistance to bacterial spot in pepper caused by *X. campestris* pv. *vesicatoria*. Tissue collapse occurs by about 12 h, 24 h and 48 h after inoculation in pepper leaves carrying the *Bs1*, *Bs2* and *Bs3* genes for resistance, respectively (Minsavage *et al.*, 1990). The induction time for establishment of the HR (i.e. the time during which protein synthesis by bacteria is necessary in the plant to cause the reaction to develop, as described by Klement and Goodman, 1967) has been found to be between 3 h and 4 h for both *Bs1* and *Bs3* (Ulla Bonas, unpublished). The signals for avirulence would appear to be generated by avirulent bacteria at the same time, but the activated response is manifest after very different 'incubation' periods. The *X.c.* pv. *vesicatoria*/pepper interaction presents a fascinating model for comparative biochemical studies of a rapid and very delayed HR. Ultrastructural studies of the *avrBs3*/*Bs3* interaction indicate that there are very few changes in the plant cell indicative of incompatibility until membrane damage becomes increasingly apparent during the second day after inoculation and cells then rapidly collapse (Brown *et al.*, 1993). The *Bs1* gene appears to be epistatic to other genes for resistance to bacterial spot (Minsavage *et al.*, 1990).

The apparent epistasis of the more rapid HR was also found in interactions between *Phaseolus* and *P. syringae* pv. *phaseolicola*, the bean halo-blight bacterium. In this interaction, five genes for avirulence (*A*) and resistance (*R*) have been postulated and three avirulence genes matching *R1*, *R2* and *R3* have been cloned (Jenner *et al.*, 1991; Mansfield *et al.*, 1994). Comparative studies of the responses of cultivars such as Guatemala (*R1* + *R3*) and A43

(R2 + R3 + R4 + R5) to races harbouring combinations of cloned *A* genes has clearly shown that the A3/R3 interaction leads to the most rapid collapse and subsequently greater accumulation of the phytoalexin, phaseollin, even when other *A* genes are also expressed (Hitchin *et al.*, 1989; Mansfield *et al.*, 1994).

Recent analyses of the *RPM1* and *RPS2* genes for resistance to *P. syringae* in *Arabidopsis* have, however, revealed that the physiologically more rapid response does not always appear as epistatic (Reuber and Ausubel, 1996; Ritter and Dangl, 1996). Experiments with these resistance genes are particularly significant as both have been cloned and are predicted to encode proteins with similar features, such as a leucine zipper, a nucleotide binding site and 14 imperfect leucine-rich repeats of approximately 24 amino acids (Mindrinos *et al.*, 1994; Grant *et al.*, 1995). Other genes that encode members of this family include the tobacco mosaic virus resistance gene *N* (Whitham *et al.*, 1994) and the flax rust resistance gene *L6* (Lawrence *et al.*, 1995; Staskawicz *et al.*, 1995). Proteins in this family have features indicating that they may represent receptors predicted by the ligand-receptor molecule for the activation of defence responses (Staskawicz *et al.*, 1995; Beynon, Chapter 19 this volume). The *RPM1* gene is also of interest because it matches different genes for avirulence, *avrRpm1* and also *avrB* (Dangl *et al.*, 1992; Grant *et al.*, 1995).

The interactions between the expression of *RPS2* and *RPM1* are summarized in Table 15.2. The *RPM1* gene confers a rapid HR in the *Arabidopsis* accession Col-0 in response to bacterial strains carrying the matching *avrRpm1* or *avrB* genes for avirulence. By contrast, with *RPS2* which is also present in Col-0, tissue collapse characteristic of the HR is not observed until about 21 h after inoculation. Differences in the responses leading to the HR were demonstrated by Reuber and Ausubel (1996). They detected two transcripts designated AIG1 and AIG2 (for *avrRpt2*-induced genes) at an early stage of the interaction between *RPS2* and *avrRpt2*, but not during expression of *RPM1*. By contrast, early accumulation of the ELI3 transcript (thought to be related to CAD) was detected only during the *avrRpm1* or *avrB* and *RPM1* interaction.

Table 15.2. *avrRpt2* action interferes with *avrRpm1* action in generation of the hypersensitive response (HR) (adapted from Ritter and Dangl, 1996).

HR timing on ^a	Pathogenic <i>Pseudomonas syringae</i> strain (DC3000 or Psm M4) expressing:				
	No <i>avr</i> gene	<i>avrRpm1</i>	<i>avrRpt2</i>	<i>avrRpm1</i> ^b + <i>avrRpt2</i>	Mix ^c
<i>RPM1/RPS2</i>	–	+ 5 h	+ 21 h	+ 21 h	+ 21 h
<i>RPM1/rps2</i>	–	+ 5 h	–	–	–

^aPlant genotypes: Col-0 (*RPM1/RPS2*); *rps2-201* (*RPM1/rps2*) has a point mutation leading to an amino acid exchange in the LRR region of *RPS2*.

^bAvirulence genes were cloned together into one plasmid, pCR105.

^cResults from mixed inoculations of equal number of *P. syringae* expressing either *avr* gene alone.

The specific accumulation of ELI3 overcomes the argument that the rapid cell death occurring during the *RPM1* response may prevent induction of AIG1 or AIG2. Different patterns of response gene expression, therefore, appear to be associated with the similar end point of resistance, i.e. cellular collapse recognized as a macroscopic HR. The assumption from these experiments is that gene expression is occurring in a cellularly homogeneous way within infiltrated tissue, but this will only be confirmed by the location of transcripts to individual cells by *in situ* hybridization.

Interference between the *RPS2* and *RPM1* responses at the level of receptor-ligand binding was indicated from experiments designed by Ritter and Dangl (1996) to dissect their finding that given expression of both *avrRpt2* and *avrRpm1* (or *avrB*) by the challenging strain of *P. syringae* it was, unexpectedly, the slower HR as controlled by *RPS2* which was expressed. Key findings were that: (i) mixtures of bacteria expressing either *avrRpm1* or *avrRpt2* induced the 'slow' HR; and (ii) apparent suppression of the *RPM1* response was also achieved with an *rps2-201* mutant derived from Col-0 (Bent *et al.*, 1994; Mindrinos *et al.*, 1994) which expresses the *RPM1* but not the *RPS2* phenotype.

The work with *RPS2* and *RPM1* represents one of the first attempts to dissect the recognition events which lead to the expression of resistance and the HR. The phenotypic variation described has been related to interference at the recognition phase of the interaction involving proteins encoded by *RPM1* and *RPS2*. The possibility that interference occurs 'downstream' of the recognition process cannot be overlooked. Analysis of mutants defective in the expression of resistance phenotypes in barley and tomato (Freialdenhoven *et al.*, 1994; Hammond-Kosack *et al.*, 1994), but with mutations in second-site loci outside the genes for resistance, indicates the requirement for additional proteins for *specific* resistance gene function. These proteins may be involved in early stages of signal transduction following elicitor/receptor binding (Hammond-Kosack and Jones, 1995).

Questions Answered or Posed?

The emphasis of the early work on the hypersensitive reaction was on the role of plant cell death in resistance to obligate parasites. Ingram (1978), in a thought-provoking discussion of this topic, emphasized the unique features of different plant/pathogen interactions. It is easy to assume that there is a common and unifying theme in gene-for-gene interactions. There is a great temptation to believe that the 'pet' interaction studied in one's own laboratory provides the answer. For instance, the speed with which IMD occurs during expression of the *Dm5/8* gene in lettuce for resistance to *B. lactucae* has provided support for the simple hypothesis that recognition leads directly to membrane damage via *de novo* protein synthesis but without mRNA synthesis in the penetrated cell. Responses requiring transcription are then activated in

surrounding cells in a scenario like that described for the HR in bean to *Colletotrichum lindemuthianum* (Bailey, 1982). Although such a simple and short chain of events may indeed operate with rapid responses such as that determined by *Dm5/8*, a more protracted and programmed form of cell death seems to occur with slower reactions such as that involving *Dm7* in lettuce and in some other plant-pathogen interactions (Ryerson and Heath, 1996).

Returning to the seven questions raised at the start of this chapter, if we simply consider the gene-for-gene interactions described above, we find very different answers within and between each plant/pathogen model. Taking each question in turn:

1. Although plant cell death is a key player in most interactions, resistance conferred by *Cf* genes in tomato has specifically been described as *not* involving a classic HR.
2. Evidence suggests that only penetrated cells undergo the HR in the *Bremia*/lettuce system, but widespread necrosis is characteristic of certain genes for powdery mildew resistance in barley and *C. fulvum* affects cells other than those with which it is in close contact. Diffusion of elicitors to groups of cells is implied by the more widespread responses.
3. The cause of cell death (irreversible membrane damage) is poorly understood. Activation of the oxidative burst has been implicated and AOS may themselves be directly damaging to membranes in the rapid HR such as that governed by *Dm5/8* (Baker and Orlandi, 1995). Alternatively, AOS such as H_2O_2 may have an indirect signalling role leading to activation of a more complex, programmed cell death (Levine *et al.*, 1994; Mehdy, 1994; Tenhaken *et al.*, 1995). Differences in transcription before cell collapse are illustrated by the responses determined by *RPS2* and *RPM1*. It is possible that the accumulation of autofluorescent phenolics may lead to the death of barley cells challenged by *E. graminis*, but similar phenolic accumulation does not cause IMD in lettuce.
4. In most interactions, cell death does precede restriction of fungal growth, but again the tomato leaf mould system proves the exception as growth is restricted in certain resistant cultivars without plant cell death.
5. Death during the rapid HR may be the major cause of inhibition of growth of obligate biotrophs, but generation of an antifungal environment by the accumulation of phytoalexins and phenolics is implicated in some studies and may be more significant with slower acting genes.
6. Evidence on the timing of transfer of signals is largely circumstantial given our lack of knowledge of elicitors. However, with *C. fulvum*, from which elicitors have been fully characterized, there is variation in the regulation of expression of *avr* genes, therefore implying differences in the likely timing of exposure of the plant to elicitors.
7. In cells undergoing the HR, many different biochemical changes can be induced. The capacity for metabolic activity is, to some extent, dictated by the

speed at which IMD occurs. This is illustrated by *Dm5/8* (rapid) and *Dm7* (slow HR) in lettuce. Some cells may be clinically executed, whereas others may suffer a long and lingering period of necrobiosis.

So far we have focused on the infection site but, given our increasing awareness of the potential for intercellular signalling between plant cells, it is worthwhile posing an eighth question: are biochemical changes confined to cells undergoing the HR or are there also *gene-specific* responses in surrounding cells? This possibility has not been considered, but it is certainly well established that surrounding cells are the main site for gene activation; for example in the production of phytoalexins and PR proteins (Schmelzer *et al.*, 1989). At present these responses appear to be secondary and dependent on the release of *non-specific* endogenous elicitors from dying cells. Moving further into the surrounding tissue and other plant parts we encounter the phenomenon of systemic acquired resistance (SAR). Salicylate has been identified as a key signal leading to the induction of SAR and there is recent evidence that, in some plants, it may also have a role in the response of some of the first cells to be challenged (Gaffney *et al.*, 1993; Delaney *et al.*, 1994; Ryals *et al.*, 1994; Mauch-Mani and Slusarenko, 1996). It would be revealing to examine the well-defined behaviour of *B. lactucae* on lettuce cultivars with the rapidly acting *Dm5/8* gene in a salicylate-free background.

We have discussed a very limited set of plant-pathogen systems, but it is clear that a wide range of variation is possible both within a single host and between plant species. Additional detail on many other interactions could have been usefully included, notably *Peronospora parasitica*/*Arabidopsis* (Holub and Beynon, 1996), *Phytophthora infestans*/potato (Tomiyama, 1967; Cuypers and Hahlbrock, 1988), *Uromyces phaseoli* var. *vignae*/cowpea (Heath, 1989) and *Puccinia recondita*/wheat (Jones and Deverall, 1977). Overall, the rather sobering conclusion to be drawn from the available evidence seems to be that there is really only one answer to each question: 'It depends on the plant and the pathogen'.

Concluding Remarks

The many interacting factors that lead to variation in phenotype during gene-for-gene interactions are outlined in Fig. 15.6. Included in this diagram is the concept of the 'recognition rheostat', a term coined by Jones and Dangl (1996) to explain the activation of different rates of response following elicitor/receptor binding. It is apparent from Fig. 15.6 that although many of the pieces in the jigsaw are common to different plant-pathogen interactions (e.g. membrane damage, cytoplasmic disorganization, phenolic deposition, phytoalexin accumulation, the oxidative burst), the way in which different pieces fit together can produce very different phenotypic outcomes.

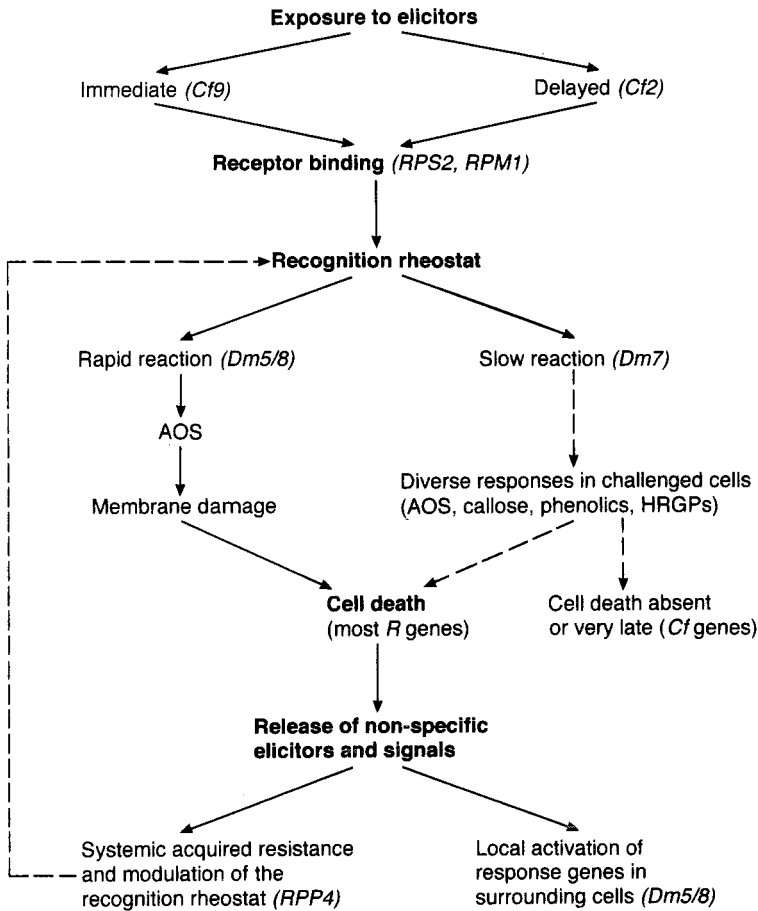


Fig. 15.6. Sources of phenotypic variation in gene-for-gene interactions. The resistance genes discussed in the text which help to illustrate key features are indicated in parentheses. Dashed lines mark indirect or less well-defined routes.

In view of the obvious differences between responses which are all used as examples of 'the HR' and the resulting confusion that occurs when broad generalizations about cause and effect are developed, we suggest that the pattern of cell death occurring during gene-for-gene interactions should be classified into HR types (HRTs) as follows; resistance genes mentioned above which confer the HRT are given in parentheses.

- HRT1: rapid reaction of penetrated cell, early restriction of fungal growth (*Dm 5/8*, *Mla1*).
- HRT2: slow reaction of penetrated cell, allowing more extensive fungal growth and trailing necrosis (*Dm7*, *RPP4*).

- HRT3: slow reaction involving death of several cells in addition to the penetrated cell (*Mla7*, *Sr7*).
- HRT4: response of cells to intercellular pathogens with no obvious penetration of the cell undergoing the HR (*RPM1*, *Cf2*).
- HRT5: cell death occurring in lesion mimics.

Note that the separate category HRT5 has been proposed for the genetically defined lesion mimics which are often put forward as models of the HR (Dietrich *et al.*, 1994) but which in practice may add yet another source of diversity and confusion!

There is little doubt that further identification of the ligands and receptors which may operate in gene-for-gene interactions will allow the biochemical steps leading from recognition to response and the inhibition of bacterial or fungal colonization to be characterized in full. Only then will the primary determinants of phenotypic variation be revealed. Perhaps a unifying mechanistic concept of the HR will emerge or, alternatively, there may be justification for further subdivision of HRTs.

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The Molecular Genetics of Specificity Determinants in Plant Pathogenic Bacteria

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Bacterial pathogens of plants are in general very limited in their host range, frequently confined to members of a single host family, genus or species. Particular isolates are often even more specialized, causing disease only in certain cultivated varieties of a particular host species. As a consequence, the vast majority of plants are resistant to attack from most bacteria.

The bacterial pathogens studied with respect to gene-for-gene host specificity generally cause spreading, water-soaked lesions on leaves where the plant host is susceptible. Resistant plants generally produce a rapid and localized collapse and necrosis of tissue in the vicinity of the entry of the pathogen. The latter was first identified in non-host plants, such as tobacco, inoculated at high density with a suspension of pathogen cells (Klement, 1963; Klement *et al.*, 1964). The so-called hypersensitive reaction (HR) has since been studied in some detail and recently it has been proposed to involve a programmed cell death (Mittler and Lam, 1996). Non-pathogens do not produce an HR, implying a link between pathogenicity and the HR. This was further confirmed by the discovery that pathogen mutants, isolated on the basis that they no longer caused disease on their susceptible host, frequently lost their ability to induce an HR in the non-host tobacco (Boucher *et al.*, 1985; Lindgren *et al.*, 1986; Malik *et al.*, 1987). Such mutants were called *hrp* (for hypersensitive reaction and pathogenicity) and have been isolated from several phytopathogens including *Pseudomonas syringae*, *Xanthomonas campestris*, *Burkholderia* (previously *Pseudomonas*) *solanacearum* and *Erwinia* spp. (Willis *et al.*, 1991; Van Gijsegem *et al.*, 1993; Bonas, 1994).

The concept of matching genes for resistance in the plant host and avirulence in the pathogen as the basis for specificity was originally proposed

by Flor (1971). This 'gene-for-gene' hypothesis has provided a useful model, resulting in the successful cloning of the first avirulence gene (*avrA*) from the soybean pathogen, *P. syringae* pv. *glycinea*. Individual clones from a gene library of the avirulent race 6 were tested in a recipient race 5, which was virulent on the cultivars Harosoy and Peking, allowing detection of clones conferring avirulence (Staskawicz *et al.*, 1984). These authors also failed to detect genes that might confer 'virulence' in race 5 on the incompatible cultivars Flambeau and Norchief, which was further evidence in support of the avirulence/resistance view of gene-for-gene interactions. Subsequent isolations of avirulence genes have relied on the same basic strategy for detection and this has confirmed the view that avirulence is a dominant function and that acquisition of a single avirulence gene is sufficient to confer a change of specificity in race/cultivar interactions. Tables 16.1 and 16.2 show the properties of avirulence genes identified and characterized to date. The cloning and sequencing of more than 30 avirulence genes from bacteria has largely failed to show why these genes confer specificity on their carriers. However, there are at last some intriguing clues, which suggest some answers.

Shortly after the isolation of the first bacterial avirulence gene involved in race/cultivar specificity, the testing of gene libraries in heterologous pathovars raised the possibility of detecting avirulence genes involved in recognition of a non-host plant (Whalen *et al.*, 1988). This led to the finding that some of these genes, such as *avrPpiA* from *P. syringae* pv. *pisi* could function both at the level of race/cultivar and at the level of pathovar/species in *P. syringae* pv. *phaseolicola* (Fillingham *et al.*, 1992). It now seems likely that the pattern of behaviour observed for individual avirulence genes is related to the availability of host-pathogen combinations in routine use, rather than to fundamental differences in function.

In the following sections, we have provided an overview of the avirulence and *hrp* genes that have been characterized in the relatively small number of different bacteria that have been studied. The avirulence genes fall broadly into two types: those that are similar to *avrBs3* and are found in the genus *Xanthomonas* and those that come mainly from *P. syringae*, which are dealt with first.

Avirulence Genes in *Pseudomonas*

The proposal of Vivian and Mansfield (1993) on the naming of avirulence genes was intended to follow, as far as possible, the standard nomenclature for bacterial genes of Demerec *et al.* (1966) and aimed to avoid confusion about the origin of an avirulence gene, while endeavouring to address the need to relate the genotype designation to that of the plant with which it interacts. It is regrettable that this nomenclature has not been more enthusiastically received, since as can be seen later in this chapter, current *laissez-faire* naming can result in considerable ambiguity. For example, there are currently four

Table 16.1. Avirulence genes cloned from *Pseudomonas syringae* pathovars.

Gene designation	Pathovar source	Interacts with host	Predicted		% GC	Genetic location	Reference
			ORF (nt)	peptide (kDa)			
<i>avrPphA</i>	<i>phaseolicola</i>	Bean	nd	nd	nd	nd	Shintaku <i>et al.</i> , 1989
<i>avrPphB1.R3</i>	<i>phaseolicola</i>	Bean/R3	801	38	48.0	Chromosome	Jenner <i>et al.</i> , 1991
<i>avrPphD</i>	<i>phaseolicola</i>	Pea	579 ^a	27 ^a	52.5 ^a	Plasmid	Wood <i>et al.</i> , 1994
<i>avrPphE1.R2</i>	<i>phaseolicola</i>	Bean/R2	1125	41	57.6	Chromosome	Mansfield <i>et al.</i> , 1994
<i>avrPphF.R1</i>	<i>phaseolicola</i>	Bean/R1	402 & 591	15 & 22	40.0 & 52.5	Plasmid ^b	Tsiamis and Mansfield, pers. comm.
<i>avrPpiA1.R2</i>	<i>pisi</i>	Pea/R2	660	24	44.0	Chr/plasmid ^c	Dangl <i>et al.</i> , 1992
<i>avrPpiB1.R3</i>	<i>pisi</i>	Pea/R3	831	31	40.0	Plasmid	Cournoyer <i>et al.</i> , 1995
<i>avrPpiC</i>	<i>pisi</i>	Bean	807	29	47.0	Chromosomal	Fillingham, 1994
<i>avrPpiD.R5</i>	<i>pisi</i>	Pea/R5	nd	nd	nd	nd	Gunn and Vivian, unpublished
<i>avrPpiE</i>	<i>pisi</i>	<i>Arabidopsis/RPS4</i>	660	24	52.0	Plasmid	Hinsch and Staskawicz, 1996
<i>avrPmaA1</i>	<i>maculicola</i>	<i>Arabidopsis/RPM1</i>	660	24	44.0	Plasmid	Dangl <i>et al.</i> , 1992
<i>avrD</i>	<i>tomato</i>	Soybean/RPG4	933	34	41.0	Plasmid	Kobayashi <i>et al.</i> , 1990
<i>avrRpt2</i>	<i>tomato</i>	Soybean/RPS2	768	28	51.5	Probably chromosomal ^d	Innes <i>et al.</i> , 1993b
<i>avrPto</i>	<i>tomato</i>	Tomato/PTO	492	18	50.5	Probably chromosomal ^e	Salmeron and Staskawicz, 1993
<i>avrE</i>	<i>tomato</i>	Soybean	nd	nd	nd	Chromosomal	Lorang and Keen, 1995
<i>avrA</i>	<i>glycinea</i>	Soybean/RPG2	2721	100	45.0	nd	Napoli and Staskawicz, 1987
<i>avrB</i>	<i>glycinea</i>	Soybean/RPG1	963	36	46.0	Chromosomal	Tamaki <i>et al.</i> , 1988
<i>avrC</i>	<i>glycinea</i>	Soybean/RPG3	1085	39	47.0	Plasmid	Tamaki <i>et al.</i> , 1988

nd = not determined.

^aM.J. Gibbon and A. Vivian, UWE-Bristol, 1996, unpublished results.^bR. Jackson, J. Mansfield and A. Vivian, UWE-Bristol and Wye College, 1995, unpublished results.^cchromosomal or plasmid-borne in different races of pv. *pisi* (Gibbon, 1994).^dR. Innes, Indiana University, 1996, personal communication.^eRonald and Staskawicz, 1988.

Table 16.2. Avirulence genes cloned from *Burkholderia* and *Xanthomonas*.

Gene designation	Pathovar source	Interacts with host	ORF (nt)	Predicted peptide (kDa)	No. of repeats ^a	Genetic location	Reference
<i>avrA</i>	<i>B. solanacearum</i>	Tobacco	nd	nd	—	nd	Carney and Denny, 1990
<i>popA</i>	<i>B. solanacearum</i>	Petunia	1002	33	—	Plasmid	Arlat <i>et al.</i> , 1994
<i>avrBs1</i>	<i>X. c. pv. vesicatoria</i>	Pepper/ <i>Bs1</i>	1335	50	—	Plasmid	Ronald and Staskawicz, 1988
<i>avrBs2</i>	<i>X. c. pv. vesicatoria</i>	Pepper/ <i>Bs2</i>	nd	nd	—	Chromosomal	Minsavage <i>et al.</i> , 1990
<i>avrBsT</i>	<i>X. c. pv. vesicatoria</i>	Pepper	nd	nd	—	Plasmid	Minsavage <i>et al.</i> , 1990
<i>avrRxv</i>	<i>X. c. pv. vesicatoria</i>	Bean/ <i>Rxv</i> , tomato	1122	42	—	Chromosomal	Whalen <i>et al.</i> , 1993
<i>avrXca</i>	<i>X. c. pv. raphani</i>	<i>Arabidopsis</i>	1851	67	—	Probably chromosomal ^b	Parker <i>et al.</i> , 1993
<i>avrBs3</i> family							
<i>avrBs3</i>	<i>X. c. pv. vesicatoria</i>	Pepper/ <i>Bs3</i>	3491	122	17.5	Plasmid	Bonas <i>et al.</i> , 1989
<i>avrBsP</i>	<i>X. c. pv. vesicatoria</i>	Tomato	nd	nd	nd	Plasmid	Canteros <i>et al.</i> , 1991
<i>avrBs3-2</i>	<i>X. c. pv. vesicatoria</i>	Tomato	3480	122	17.5	Plasmid	Bonas <i>et al.</i> , 1993
<i>avrBn</i>	<i>X. c. pv. malvacearum</i>	Cotton	nd	nd	nd	Chromosomal	Gabriel <i>et al.</i> , 1986
<i>avrb6</i>	<i>X. c. pv. malvacearum</i>	Cotton/ <i>B1</i>	nd	nd	13.5 ^c	Plasmid	De Feyter <i>et al.</i> , 1993
<i>avrB4</i>	<i>X. c. pv. malvacearum</i>	Cotton/ <i>B1</i> , <i>B4</i>	nd	nd	19.0	Plasmid	De Feyter <i>et al.</i> , 1993
<i>avrb7</i>	<i>X. c. pv. malvacearum</i>	Cotton	nd	nd	19.0	Plasmid	De Feyter <i>et al.</i> , 1993
<i>avrBln</i>	<i>X. c. pv. malvacearum</i>	Cotton	nd	nd	21.0	Plasmid	De Feyter <i>et al.</i> , 1993
<i>avrB101</i>	<i>X. c. pv. malvacearum</i>	Cotton	nd	nd	22.5 ^c	Plasmid	De Feyter <i>et al.</i> , 1993
<i>avrB102</i>	<i>X. c. pv. malvacearum</i>	Cotton/ <i>B1</i>	nd	nd	18.0	Plasmid	De Feyter <i>et al.</i> , 1993
<i>avrB103</i>	<i>X. c. pv. malvacearum</i>	Cotton	nd	nd	nd	Chromosomal	Yang <i>et al.</i> , 1996
<i>avrB104</i>	<i>X. c. pv. malvacearum</i>	Cotton	nd	nd	nd	Chromosomal	Yang <i>et al.</i> , 1996
<i>avrB5</i>	<i>X. c. pv. malvacearum</i>	Cotton	nd	nd	nd	Chromosomal	Yang <i>et al.</i> , 1996
<i>avrxs5</i>	<i>X. o. pv. oryzae</i>	Rice/ <i>Xa-5</i>	nd	nd	nd	nd	Hopkins <i>et al.</i> , 1992
<i>avrXa7</i>	<i>X. o. pv. oryzae</i>	Rice/ <i>Xa-7</i>	nd	nd	25.0	nd	Hopkins <i>et al.</i> , 1992
<i>avrXa10</i>	<i>X. o. pv. oryzae</i>	Rice/ <i>Xa-10</i>	3306	116	15.5	Chromosomal ^d	Hopkins <i>et al.</i> , 1992
<i>pthA</i>	<i>X. citri</i>	Bean, cotton	3491	122	17.5	nd	Swarup <i>et al.</i> , 1992

nd = not determined.

^anumber of 102 bp direct repeats in the central region of the genes in the *avrBs3* family.^bM.J. Daniels, IPSR, Norwich, 1996, personal communication.^cD.W. Gabriel, University of Florida, 1996, personal communication.^dKelemu and Leach, 1990.

genes designated *avrA* from *P. syringae* pv. *glycinea* (Staskawicz *et al.*, 1984), *P. s.* pv. *tomato* (Kobayashi *et al.*, 1989), *P. syringae* pv. *phaseolicola* (Shintaku *et al.*, 1989) and *B. solanacearum* (Carney and Denny, 1990), three of which encode different specificities.

Pseudomonas syringae* pv. *phaseolicola

Isolates of *P. syringae* pv. *phaseolicola* cause halo-blight of bean and currently comprise nine races distinguished by their differential interaction with eight bean cultivars (Teverson *et al.*, 1997). Five matching genes for avirulence (A) in the pathogen and resistance (R) in the host have been postulated (Table 16.3), of which three of these gene pairs have so far been defined by the isolation of the corresponding A gene. However, the first avirulence gene (*avrA*) to be described for *P. syringae* pv. *phaseolicola* (Shintaku *et al.*, 1989) does not correspond to any of the predicted specificities of the gene-for-gene scheme (G. Tsiamis and J. Mansfield, Wye College, 1996, personal communication).

The second gene isolated is currently designated *avrPphB1.R3* (previously *avrPph3*) and, as indicated by the nomenclature of Vivian and Mansfield (1993), this gene matches the R3 gene in bean and therefore corresponds to the putative A3 gene (Table 16.3; Hitchin *et al.*, 1989). A homologue of *avrPphB1*, designated *avrPphB2.R3*, was isolated from race 4; the two alleles share complete sequence identity within their open reading frames (ORFs) (Jenner *et al.*, 1991). It has recently been shown that *avrPphB* is regulated by

Table 16.3. Gene-for-gene relationship between bean cultivars and races of *Pseudomonas syringae* pv. *phaseolicola* (Teverson *et al.*, 1997).

		<i>P. syringae</i> pv. <i>phaseolicola</i> race								
		1	2	3	4	5	6	7	8	9
Bean cultivar	Resistance gene	Avirulence gene								
		1	2	3	4	5	6	7	8	9
		1	2	3	4	5	6	7	8	9
		1	2	3	4	5	6	7	8	9
		1	2	3	4	5	6	7	8	9
Canadian Wonder	1	+	+	+	+	+	+	+	+	+
A52 (ZAA54)	4	+	+	+	+	+	+	+	+	+
Tendergreen	3	+	+	+	+	+	+	+	+	+
Red Mexican UI3	4	+	+	+	+	+	+	+	+	+
1072	2	+	+	+	+	+	+	+	+	+
A53 (ZAA55)	3	4	+	+	+	+	+	+	+	+
A43 (ZAA12)	2	3	4	5	+	+	+	+	+	+
Guatemala 196-B	1	3	4	+	+	+	+	+	+	+

+ susceptible response; – resistant response; . gene absent.

hrpL (J. Mansfield, Wye College, 1996, personal communication). This gene also functions in certain isolates of other pathovars to confer avirulence toward pea, *Arabidopsis* and soybean (Fillingham *et al.*, 1992; Simonich and Innes, 1995). Several other examples (see below) are now known where avirulence genes interact with functional homologues of resistance genes in a number of plant hosts from taxonomically disparate genera.

A second avirulence gene was isolated from race 4 of *P. syringae* pv. *phaseolicola* and shown to correspond to the putative A2 gene (Table 16.3; Mansfield *et al.*, 1994). This gene, designated *avrPphE1.R2* (Table 16.1) is situated at the left-hand end of the cluster of *hrp* genes defined by Rahme *et al.* (1991) and was found to be adjacent to a newly discovered gene, designated *hrpY* in *P. syringae* pv. *phaseolicola* (Mansfield *et al.*, 1994). This new locus is homologous to the *hrpK* gene from *P. syringae* pv. *syringae* (Xiao *et al.*, 1994). Use of a DNA probe constructed from an internal sequence of *avrPphE1* showed that homologues of the gene were present in all races including five that were virulent on R2-containing cultivars. No polymorphism was detected in digests with *HindIII*, *EcoRI* and *PstI* and identical hybridizing fragments were also detected in an isolate of *P. syringae* pv. *tabaci*. The presence of apparently non-functional alleles of *avrPphE* in races lacking the A2 phenotype (Mansfield *et al.*, 1994) was in direct contrast to the results with *avrPphB* and *avrPphF*, which were found only in isolates avirulent on R3 and R1 genotypes, respectively (Jenner *et al.*, 1991; G. Tsiamis and J.W. Mansfield, Wye College, 1996, personal communication). This conserved chromosomal gene has the highest G + C content yet found among *P. syringae* avirulence genes, approaching the overall G + C content of the *P. syringae* genome (Table 16.1).

Disruption of *avrPphE* by *Tn3-gus* in races 6 and 7 did not appear to affect pathogenicity in pod tests, but did prevent induction of an HR by race 7 on genotypes carrying R2, such as cultivar A43, where the reaction was reduced to a null (Mansfield *et al.*, 1994). It is interesting to note that in *P. syringae* pv. *tomato*, an unrelated avirulence gene, *avrE*, is located adjacent to the *hrpRS* region at the other end of the corresponding *hrp* cluster (Lorang and Keen, 1995).

The most recent avirulence gene in *P. syringae* pv. *phaseolicola* was isolated from race 5 and designated *avrPphF.R1*. Sequencing has identified two open reading frames, both of which are required for the A1 phenotype (Table 16.3; G. Tsiamis and J.W. Mansfield, Wye College, 1996, personal communication).

Two further genes, designated *avrPphC* (Yucel *et al.*, 1994b) and *avrPphD* (Wood *et al.*, 1994), interact exclusively with non-host plants. The first of these, *avrPphC*, is plasmid-borne and linked within 5 kb to an allele of *avrD* from *P. syringae* pv. *tomato*. The gene *avrPphC* is over 99% homologous in its DNA sequence to *avrC* from race 0 of *P. syringae* pv. *glycinea* (Tamaki *et al.*, 1988) and the predicted peptides differ by just two amino acid substitutions. Phenotypically, the genes were identical in their interactions with soybean cultivars bearing the matching *RPG3* resistance gene (Yucel *et al.*, 1994b).

Cloned DNA from a 150 kb plasmid in the *P. syringae* pv. *phaseolicola* race 4 isolate 1302A conferred avirulence toward pea in *P. syringae* pv. *lisi*; sub-cloning and transposon mutagenesis identified two regions of DNA that were required for the avirulence phenotype (Wood *et al.*, 1994), although it now appears that region II may be involved in stability of the cloned DNA (M.J. Gibbon and A. Vivian, UWE-Bristol, 1995, unpublished results).

Pseudomonas syringae* pv. *lisi

Bacterial blight of pea, caused by *P. syringae* pv. *lisi*, is a seed-borne disease of worldwide occurrence. Isolates comprise seven naturally occurring races, which are distinguished by their interactions with eight pea differential cultivars. A gene-for-gene scheme proposes up to six matching A and R gene pairs (Table 16.4; Bevan *et al.*, 1995). In addition, two spontaneous variants obtained in the laboratory present novel host specificities, which could be regarded as two further races expressing the genes A1/A4 and A4/A5, although neither has been isolated from the wild to date (Bavage *et al.*, 1991; C. Gunn, M. Gibbon, J.D. Taylor and A. Vivian, UWE-Bristol and HRI, Well-sbourne, 1995, unpublished results). Two of the possible six avirulence genes have thus far been isolated and characterized, together with the isolation of a cosmid library clone that appears to carry the putative A5 gene (C. Gunn and A. Vivian, UWE-Bristol, 1995, unpublished results).

Table 16.4. Gene-for-gene relationship between pea cultivars and races of *Pseudomonas syringae* pv. *lisi*. (based on Bevan *et al.*, 1995).

Pea cultivar	Resistance gene						<i>P. syringae</i> pv. <i>lisi</i> race						
							1	2	3	4	5	6	7
							{ 1 2 3 4 5 6?	1	.	.	.	.	.
								.	2	.	2	.	2
								3	.	3	.	.	3
								4	.	.	4	4	4
								.	.	.	5	.	.
								6?	.	.	6?	.	.
Kelvedon Wonder	.	.	.	.	.	.	+	+	+	+	+	+	+
Early Onward	.	2	.	.	.	.	+	–	+	+	–	+	–
Belinda	.	.	3	.	.	.	–	+	–	+	+	+	–
Hurst Greenshaft	.	.	.	4	.	6?	–	+	+	–	–	+	–
Partridge	.	.	3	4	.	.	–	+	–	–	–	+	–
Sleaford Triumph	.	2	.	4	.	.	–	–	+	–	–	+	–
Vinco	1	2	3	.	5	.	–	–	–	+	–	+	–
Fortune	.	2	3	4	.	.	–	–	–	–	–	+	–

+ susceptible response; – resistant response; ? gene probably present; . gene absent.

Initially, the gene corresponding to A2 in race 2 (Table 16.4) was isolated and shown to interact with a single R2 resistance gene in pea (Vivian *et al.*, 1989). The gene was subsequently designated *avrPpiA1.R2* and shown to be almost identical to a gene from *P. syringae* pv. *maculicola*, designated *avrRpm1* (also *avrPmaA1.RPM1*) (Dangl *et al.*, 1992). Homologues were detected by hybridization to a gene-specific probe only in those races of *P. syringae* pv. *pisi* expressing the A2 phenotype and while in race 2 the gene is chromosomally located, homologues in races 5 and 7 are plasmid-borne (Gibbon, 1994).

The plasmid-borne gene corresponding to A3 was isolated from race 3 and designated *avrPpiB1.R3* (Table 16.4; Bavage *et al.*, 1991; Cournoyer *et al.*, 1995). It matches a single R3 resistance gene in pea and plasmid-borne homologues have been detected in races 1 and 7. Homologues of this gene were also detected in isolates of *P. syringae* pv. *tomato*, *P. syringae* pv. *phaseolicola* and *P. syringae* pv. *maculicola*, although it is unclear whether these are functional. The finding that *avrPpiB* homologues are present in races 1, 5 and 6 of *P. syringae* pv. *phaseolicola* does not accord with any of the avirulence loci in the proposed gene-for-gene interaction with bean (Table 16.3), hence it would appear that either those bean cultivars tested do not possess a resistance gene functionally homologous to the R3 gene of pea or that the expression of this gene is strain-dependent, as has been found for the *avrPphB* gene in *Arabidopsis* (Simonich and Innes, 1995).

Recently a cosmid clone from a race 5 library has been shown to confer avirulence toward pea cultivar Vinco, but not to Sleaford Triumph, suggesting that it harbours the determinant corresponding to A5 (Table 16.4) and that the resistance gene R5 is absent from cv. Sleaford Triumph (C. Gunn, A. Vivian and J.D. Taylor, UWE-Bristol and HRI, Wellesbourne, 1995, unpublished results).

In a search for avirulence genes in *P. syringae* pv. *pisi*, which matched non-host resistance genes in bean, Fillingham (1994) obtained a cosmid clone from the race 5 isolate 974B gene library, which conferred avirulence in *P. syringae* pv. *phaseolicola* toward all bean cultivars; the gene responsible has provisionally been designated *avrPpiC* (J. Fillingham, J.R. Wood, J.W. Mansfield and A. Vivian, Wye College and UWE-Bristol, 1994, unpublished results).

A further avirulence gene from *P. syringae* pv. *pisi*, designated *avrRps4*, has recently been described by Hinsch and Staskawicz (1996). This gene, which we would prefer to call *avrPpiE.RPS4*, was plasmid-borne in race 1 strain 151 and was also shown to be conserved on two *HincII* fragments in all the type races, except the race 5 isolate 974B. Strain 151 induced an HR in *Arabidopsis* Po-1; the clone conferred genotype specificity in *P. syringae* pv. *tomato* strain DC3000 towards Po-1, Ws-0 and 18 other host accessions, but not to RLD. This enabled genetic analysis between susceptible and resistant accessions which showed that Ws-0 carries a single dominant resistance gene (*RPS4*) that matches *avrPpiE.RPS4* (Hinsch and Staskawicz, 1996). This gene did not confer avirulence in either *P. syringae* pv. *pisi* or *P. syringae* pv. *syringae* toward the pea

differential cultivars (M.J. Gibbon and A. Vivian, UWE-Bristol, 1994, unpublished results).

Pseudomonas syringae* pv. *maculicola

Towards the end of the 1980s, researchers realized the potential of using the so-called model plant species, *Arabidopsis thaliana*, for molecular analyses of resistance genes that correspond in a specific manner with known avirulence genes in bacterial pathogens. Isolates from several pathovars of *P. syringae* were demonstrated as being capable of causing disease in *Arabidopsis*, including *P. syringae* pv. *maculicola*, *P. syringae* pv. *tomato* and *P. syringae* pv. *pisi* (Davis *et al.*, 1991; Dong *et al.*, 1991; Whalen *et al.*, 1991).

A plasmid-borne gene, designated *avrRpm1* (synonym *avrPmaA1*), was isolated from the *P. syringae* pv. *maculicola* isolate m2 and shown to confer avirulence toward *Arabidopsis* accession Col-0 (Debener *et al.*, 1991). Subsequent DNA sequence analysis and hybridization showed the gene to be homologous to *avrPpiA1* from *P. syringae* pv. *pisi* and to *avrPmaA2* in *P. syringae* pv. *maculicola* isolate 791. The matching resistance gene, *RPM1*, in *Arabidopsis* was shown to condition resistance to bacterial strains carrying either *avrRpm1* or *avrPpiA* (Dangl *et al.*, 1992) and recently, an allele or closely linked gene at the resistance locus, *RPG1*, in soybean has been shown to interact in a gene-for-gene manner with *avrRpm1*, in addition to its interaction with *avrB* (Ashfield *et al.*, 1995).

Marker-exchange disruption of the *avrRpm1* gene in isolate m2 resulted in loss of the ability to induce an HR in the *RPM1*-carrying accession Col-0 (as expected) and also loss of ability to produce disease symptoms or grow in compatible accessions such as Mt-0, Nd-0 and Fe-1. Transposon insertions confirmed the role of *avrRpm1* in virulence of *P. syringae* pv. *maculicola* m2 toward *Arabidopsis*. However, no visible effects of the marker-exchanged mutants were seen in the HR induced on pea and bean, which carry homologues of *RPM1*, nor in the pathogenicity on radish and turnip, which are both hosts for isolate m2 (Ritter and Dangl, 1995).

Pseudomonas syringae* pv. *tomato

P. syringae pv. *maculicola* and *P. syringae* pv. *tomato* are phenotypically very similar and have overlapping host ranges (Hendson *et al.*, 1992; Takikawa *et al.*, 1994); their taxonomic position remains uncertain, although Cuppels and Ainsworth (1995) recently noted, on the basis of pathogenicity and carbon utilization, that a strain of *P. syringae* pv. *tomato* called DC3000 more closely resembles *P. syringae* pv. *maculicola* than other strains of *P. syringae* pv. *tomato*.

An avirulence gene isolated by Kobayashi *et al.* (1989) was shown to be virtually identical to *avrA* from *P. syringae* pv. *glycinea* race 6 (Staskawicz *et al.*,

1984; Napoli and Staskawicz, 1987) and should perhaps be designated *avrA2*. Another gene, *avrD*, was isolated from *P. syringae* pv. *tomato* strain PT23 (Kobayashi *et al.*, 1989). DNA sequencing of a 5.6 kb region from the 83 kb plasmid pPT23B (Murillo *et al.*, 1994) detected five open reading frames (ORFs), the first of which corresponded to *avrD* (Kobayashi *et al.*, 1990). While the protein product of *avrD* did not function as an elicitor, its expression in *P. syringae* or *Escherichia coli* resulted in the detection in culture fluids of a low-molecular-weight elicitor (Keen *et al.*, 1990). The elicitor(s), which are also the subject of Noel Keen (Chapter 20 this volume) was subsequently shown to comprise two related molecules called syringolides (Midland *et al.*, 1993; Smith *et al.*, 1993).

Three new alleles of *avrD* were cloned in a search for possible homologues (Yucel *et al.*, 1994a). Failure to adopt the use of allele numbers for the unambiguous assignment of related forms of a single gene led to a cumbersome nomenclature in this instance and we propose the adoption of the allele designations (Table 16.5). The two most divergent alleles were isolated from 90 and 75 kb indigenous plasmids in *P. syringae* pv. *lachrymans* and appear to have been designated alleles 1 (*avrD3*) and 2 (*avrD4*), respectively, while the third (*avrD5*) was from *P. syringae* pv. *phaseolicola*. The relationships between these alleles permitted their division into two groups, class I, comprising *avrD1* (the original *avrD* from *P. syringae* pv. *tomato*) and *avrD3*, and class II, comprising *avrD2* (from *P. syringae* pv. *glycinea*), *avrD4* and *avrD5* (Yucel *et al.*, 1994a). In a subsequent paper, the two classes of gene product were shown to have specificity for their substrates, class I utilizing both β -hydroxyoctanoic acid and β -hydroxydecanoic acid, while class II utilized only β -hydroxyoctanoic acid (Yucel *et al.*, 1994c).

avrRpt2 was simultaneously isolated by Dong *et al.* (1991) and Whalen *et al.* (1991) from the *P. syringae* pv. *tomato* strain JL1065 and shown to match a single resistance gene, *RPS2*, in *Arabidopsis* Col-0. On transfer to race 4 of *P. syringae* pv. *glycinea*, *avrRpt2* conferred cultivar-specific avirulence toward soybean cultivars Centennial, Flambeau and Harosoy, suggesting the likelihood that these cultivars harbour a functional homologue of the Col-0

Table 16.5. Some alleles of avirulence genes cloned from *Pseudomonas syringae*.

Gene designation	Cloned from pv.	Allele of	Cloned from pv.	Reference
<i>avrA</i>	<i>tomato</i>	<i>avrA</i>	<i>glycinea</i>	Kobayashi <i>et al.</i> , 1989
<i>avrPphC</i>	<i>phaseolicola</i>	<i>avrC</i>	<i>glycinea</i>	Yucel <i>et al.</i> , 1994b
<i>avrD2</i>	<i>glycinea</i>	<i>avrD</i>	<i>tomato</i>	Yucel <i>et al.</i> , 1994a
<i>avrD3</i>	<i>lachrymans</i>	<i>avrD</i> (allele 1)	<i>tomato</i>	Yucel <i>et al.</i> , 1994a
<i>avrD4</i>	<i>lachrymans</i>	<i>avrD</i> (allele 2)	<i>tomato</i>	Yucel <i>et al.</i> , 1994a
<i>avrD5</i>	<i>phaseolicola</i>	<i>avrD</i>	<i>tomato</i>	Yucel <i>et al.</i> , 1994a
<i>avrPmaA1</i>	<i>maculicola</i>	<i>avrPpiA</i>	<i>pisi</i>	Dangl <i>et al.</i> , 1992
<i>avrPmaA2</i>	<i>maculicola</i>	<i>avrPpiA</i>	<i>pisi</i>	Dangl <i>et al.</i> , 1992

resistance gene, *RPS2*. The bean cultivar Bush Blue Lake was also shown to interact with *avrRpt2* in *P. syringae* pv. *phaseolicola*, consistent with the presence of a functional homologue of *RPS2* in bean (Innes *et al.*, 1993b). DNA sequence analysis of *avrRpt2* revealed a putative hydrophilic protein, comparable in size but unrelated in sequence to many other avirulence genes.

A further gene from *P. syringae* pv. *tomato*, designated *avrE*, confers avirulence on race 4 of *P. syringae* pv. *glycinea* on all soybean cultivars. This activity required an extensive region of chromosomal DNA (approximately 9–11 kb) and was located next to the right-hand end of the *hrp* gene cluster close to *hrpRS*. DNA sequence analysis revealed four transcriptional units, designated II to V and of these units III and IV were essential for *avrE* function. Marker-exchange mutagenesis in *P. syringae* pv. *tomato* strain DC3000 in each of the four units II to V did not affect virulence toward tomato or HR on tobacco and soybean (Lorang and Keen, 1995). The particular region studied by Lorang and Keen (1995) corresponds to the region used by Henderson *et al.* (1992) to investigate the taxonomy of *P. syringae* pv. *maculicola*/*P. syringae* pv. *tomato* and related isolates and this region was highly conserved both in these isolates and in nine other *P. syringae* pathovars.

It has often been suggested that because some avirulence genes appear to have dual function, both at the race/cultivar level and also at the pathovar/host species level, that non-host resistance results from the additive effects of several individual avirulence genes (e.g. Dangl, 1994). Recently, Lorang *et al.* (1994) set out to test this hypothesis in *P. syringae* pv. *tomato* strain PT23, which is pathogenic on tomato but induces an HR on all soybean cultivars. They introduced mutations into the genes *avrA*, *avrE* and *avrPto* in a cured derivative of PT23 that lacked the plasmid bearing *avrD*, thus creating a strain (MXADEP) that had all four genes inactivated compared with the wild-type PT23. The mutant MXADEP retained the ability to induce an HR on all soybean cultivars at an inoculum density of 10^{-8} cfu ml⁻¹ and in tobacco, indicating that the four avirulence genes do not contribute to the non-host resistance of soybean and tobacco to *P. syringae* pv. *tomato* strain PT23 (Lorang *et al.*, 1994).

The avirulence gene, *avrPto*, matches the resistance gene *PTO* in tomato and also confers avirulence in *P. syringae* pv. *glycinea* toward the soybean cv. Centennial. Mutation of *avrPto* in *P. syringae* pv. *tomato* did not result in virulence toward tomato (Ronald *et al.*, 1992). Lorang *et al.* (1994) further observed that strain MXADEP retained the ability to induce an HR in tomato cv. Peto 76R, which contains the resistance gene *PTO*.

Pseudomonas syringae* pv. *glycinea

The first avirulence gene (*avrA*) to be isolated was cloned from the soybean pathogen *P. syringae* pv. *glycinea* race 6 and was not detected in other races.

DNA fragments flanking the gene showed multiple hybridizing bands in races 1, 4, 5 and 6, suggesting the possibility of mobility for this region of DNA (Staskawicz *et al.*, 1984). This gene matches the RPG2 resistance gene in soybean (Keen and Buzzell, 1991).

Two genes, *avrB* and *avrC*, from race 0 of *P. syringae* pv. *glycinea* match the RPG1 and RPG3 resistance genes, respectively (Keen and Buzzell, 1991). The two genes had repeated DNA in their flanking regions (Staskawicz *et al.*, 1987) and a low overall G + C content of 46 and 47%, respectively (Table 16.1). Although sharing 42% identity of amino acids in their respective peptides, the phenotypes produced *in planta* differed: *avrB* gave a more rapid and necrotic HR than *avrC* (Tamaki *et al.*, 1988).

Recombinants formed from *avrB* and *avrC* showed that the central regions were required for specificity of avirulence gene activity, but the flanking regions of the ORFs were interchangeable. Chimeric genes did not result in any novel avirulence phenotypes, suggesting that the genes have a catalytic function (Tamaki *et al.*, 1991). These results were also similar to those obtained with chimeric *nodE* genes in *Rhizobium leguminosarum* and *R. trifolii*, except that *nodE* determines a positive function determining the ability to colonize the host (Spaink *et al.*, 1989).

Behaviour of *avrB* in *Arabidopsis* and other host plants

The introduction of *avrA* and *avrD* from *P. syringae* pv. *tomato* and *avrB* and *avrC* from *P. syringae* pv. *glycinea* into *P. syringae* pv. *maculicola* strain 4326 and inoculation into *Arabidopsis* Col-0 showed that only *avrB* was recognized by a resistance locus in this accession (Wanner *et al.*, 1993). Innes *et al.* (1993a) reported that *avrB* matched a resistance gene, designated RPS3, in *Arabidopsis*, which they were unable to separate from the resistance gene RPM1 (Grant *et al.*, 1995) in a cross of resistant and susceptible accessions. In an attempt to resolve the question as to whether these two resistance genes were the same, Bisgrove *et al.* (1994) obtained 12 point mutants that were susceptible to strains carrying *avrB*; all of these mutants were also susceptible to strains carrying *avrRpm1* (Dangl *et al.*, 1992). No mutants were recovered that had lost only the 'RPS3' activity; thus, the two genes are the same (Bisgrove *et al.*, 1994).

Interestingly, *avrB* also functions in *P. syringae* pv. *pisi* toward pea in a cultivar-specific fashion. Given that in *Arabidopsis* the genes *avrB* and *avrRpm1* (which is virtually identical to *avrPpiA1*) interact with a single resistance gene (Bisgrove *et al.*, 1994), it appears that in pea the situation may be different. Here, the pea cultivars Early Onward, Martus and Vinco all carry an R2 resistance gene (Table 16.4) and thus gave an HR with *P. syringae* pv. *pisi* race 4 carrying *avrPpiA1*, whereas when *avrB* was introduced into race 4, an HR was only observed with cv. Vinco. A cross of Vinco (R2 and putative

RB) × Kelvedon Wonder (universal susceptible) showed that resistance matching the two avirulence genes clearly segregated (M.J. Gibbon and A. Vivian, UWE-Bristol, 1996, unpublished results). This suggests that matching of avirulence and resistance genes does not always result from the same signal perception in different host plants, with implications for the manipulation of resistance by heterologous transfer between species.

Avirulence Genes in *Burkholderia solanacearum*

An avirulence activity, confusingly designated *avrA*, was located on a 2 kb region of DNA from *B. solanacearum* strain AW1 (derived from the wild-type AW), which was pathogenic to tomato and eggplant, but gave an HR on tobacco. When the cloned gene was introduced into a second strain NC252 (derived from the wild-type NC25), which was pathogenic on all three hosts, it conferred the ability to induce an HR in tobacco while remaining pathogenic on tomato and eggplant. Marker-exchange mutagenesis of *avrA* in AW1 resulted in the loss of the HR toward tobacco but did not result in wilting symptoms typical of a pathogenic reaction, suggesting that further genes are involved in the limitation of host range on tobacco. The gene was not dependent on nutritional status for its expression, suggesting that it was constitutively expressed (Carney and Denny, 1990).

A novel type of protein with elicitor activity was isolated by Arlat *et al.* (1994). PopA1 and its related truncated peptide PopA3 elicit an HR-like reaction in the non-host tobacco, but none in the normal host, tomato. The gene specifying both peptides, designated *popA*, maps just outside of the *hrp* cluster but belongs to the *hrp* regulon. A search of the sequence databases failed to find any homology to previously determined sequences. Petunia cultivars that were sensitive to PopA1 were resistant to the *B. solanacearum* wild-type strain, while those that were insensitive gave no reaction. Mutants of *popA* retain full virulence toward tomato and still induce an HR in tobacco and resistant petunia, indicating that *popA* is not a *hrp* gene, but resembles an avirulence gene. Confirmation of the phenotype awaits the discovery of a susceptible petunia host. PopA was similar in size, heat stability and glycine-rich composition to the harpins from *P. syringae* and *E. amylovora*, but differed from them in a number of structural features (Arlat *et al.*, 1994).

Avirulence Genes in *Xanthomonas*

Xanthomonas campestris* pv. *raphani

An avirulence gene, designated *avrXca*, was isolated from *X. campestris* pv. *raphani* strain 1067, which conferred avirulence to *Arabidopsis* but not to

turnip in *X. campestris* pv. *campestris* (Parker *et al.*, 1993). Marker-exchange mutagenesis in strain 1067 did not result in compatibility toward *Arabidopsis* Col-0 or affect the interaction with turnip, suggesting either that more avirulence/R gene pairs were operating or that mutation of *avrXca* reduced pathogen fitness. Sequencing revealed a consensus *hrp* box, 40 bp upstream of the putative start codon and the N-terminal region had features of a prokaryotic signal peptide, suggesting translocation of the protein across the bacterial inner membrane into the periplasmic space or possible secretion to the outside of the cell. The function of *avrXca* was not *hrp*-dependent or related to the nutritional status of the bacteria. The use of an internal gene probe detected hybridizing bands in *X. campestris* pvs *campestris*, *raphani*, *armoraciae*, *aberrans*, *vitians*, *vesicatoria* and *malvacearum*, but none in pvs *holcicola* and *graminis*. The presence of hybridizing sequences in *X. campestris* pv. *campestris* strain 8004 suggested the presence of a non-functional homologue; mutation of this homologue did not affect virulence toward *Brassica* or *Arabidopsis* (M.J. Daniels, IPSR, Norwich, 1996, personal communication). The gene did not confer a rapid and necrotic HR in *Arabidopsis* and gave a similar phenotype after transfer to *X. campestris* pv. *armoraciae* on *Arabidopsis* Col-0, indicating that the slow HR and absence of necrosis were typical of the gene rather than being due to the genetic background of the recipient strain (Parker *et al.*, 1993).

Xanthomonas campestris* pv. *vesicatoria

Isolates of *X. campestris* pv. *vesicatoria*, a pathogen of pepper and tomato causing a spot disease on both leaves and fruit, have been assigned to three races on the basis of their interactions with near-isogenic lines of pepper (Minsavage *et al.*, 1990). Within each race, isolates could also be distinguished by their ability to cause disease in tomato (Canteros *et al.*, 1991). Minsavage *et al.* (1990) recognized three groups of *X. campestris* pv. *vesicatoria* based on their response to infection of the tomato cv. Walter and three pepper lines carrying the resistance genes *Bs1*, *Bs2* and *Bs3*. On this basis, isolates which were only virulent on tomato were designated XcvT, those not virulent on tomato were designated XcvP, and those which showed specificity on pepper lines but were also virulent on tomato were designated XcvPT. XcvT and XcvP were all race 1 isolates, whereas XcvPT belonged to either race 2 or race 3.

Resistance to race 2 of *X. campestris* pv. *vesicatoria* was due to a gene *Bs1* carried by the pepper line ECW10R. Matching avirulence to this resistance was shown to be linked to copper-resistance on a 200 kb conjugative plasmid (Stall *et al.*, 1986). The gene responsible, *avrBs1*, which was obtained from XcvPT race 2, strain 81-23 (Swanson *et al.*, 1988) showed about 47% overall homology to the carboxyl-terminal region of the *P. syringae* pv. *glycinea* gene, *avrA*, and 86% homology over a region of 49 amino acids. The homology stopped abruptly at the end of *avrA* and there was no detectable homology at

the DNA level. *P. syringae* pv. *glycinea* did not induce an HR in the pepper line ECW10R, which carries the matching *Bs1* resistance gene (Ronald and Staskawicz, 1988).

Spontaneous race change mutants of *X. campestris* pv. *vesicatoria* race 2 that had lost avirulence toward pepper cultivars carrying the resistance gene *Bs1* were found to have insertions in the *avrBs1* gene. The transposable element responsible, designated IS476, resulted in high frequency of around 5×10^{-4} of virulent mutants in race 2 (Kearney *et al.*, 1988; Swanson *et al.*, 1988). Very few examples of race change have been investigated at the molecular level, but it is perhaps significant that loss of avirulence in this case was associated with disruption of the gene by a transposable element, rather than complete loss through curing of the plasmid.

Both *avrBs2* and *avrBs3* (see later) were obtained from race 1 of *X. campestris* pv. *vesicatoria*. The *avrBs2* gene conferred specific avirulence toward the *Bs2* resistance gene and was shown to be chromosomally located. Unlike the genes *avrBs1* and *avrBs3*, *avrBs2* is highly conserved among all strains of *X. campestris* pv. *vesicatoria* examined and among many other pathovars of *X. campestris*. Mutants lacking *avrBs2* activity were shown to be due to single-base changes in the gene since fragments of the same size were detected using a gene-specific probe in both wild type and mutant (Minsavage *et al.*, 1990). Mutation at the *avrBs2* locus in *X. campestris* pv. *vesicatoria* resulted in reduced virulence, reflected in a reduced growth rate in the normal host pepper, while mutation of a homologous gene in *X. campestris* pv. *alfalfae* showed a similar growth reduction in alfalfa. This confirmed the importance of *avrBs2* to the fitness of both these pathogens (Kearney and Staskawicz, 1990).

Minsavage *et al.* (1990) isolated an avirulence gene, designated *avrBsT* from *X. campestris* pv. *vesicatoria* race 1 XcvT strain 75-3, which conferred avirulence on a race 2 strain toward the pepper line ECW. This gene was located on a 41 kb plasmid and mutants were readily obtained that had lost both the plasmid and the avirulence activity toward ECW. This spontaneous loss of *avrBsT* suggested that loss or inactivation of a single avirulence gene might extend the host range to 'non-hosts' for certain bacteria (Minsavage *et al.*, 1990). This conclusion is contentious, however, since the host range of many *X. campestris* pv. *vesicatoria* isolates clearly includes both pepper and tomato. The *avrBsT* gene sequence is not recorded in the databases.

The first attempt to examine whether non-host resistance between bacteria and plants was fundamentally similar to that seen in race/cultivar-specific resistance was in *X. campestris* pv. *vesicatoria* tomato race 1 XcvT strain 75-3. This strain gives an HR on bean, soybean, cowpea, alfalfa and cotton. Screening of a gene library in the related *X. campestris* pv. *phaseoli* on bean resulted in the isolation of an avirulence gene, *avrRxv* (Whalen *et al.*, 1988). Subsequently, the same gene was again isolated from the library by screening on tomato cv. Hawaii 7998, showing that this gene was involved in both host

and non-host resistance. Sequence analysis of *avrRxv* revealed a putative hydrophilic peptide with no membrane-spanning domains or secretion signals. This peptide had some similarity to RNA polymerase subunits, suggesting a possible DNA-binding function (Whalen *et al.*, 1993).

Resistance in bean segregated in a gene-for-gene manner as a partially dominant gene present in cv. Sprite toward *X. campestris* pv. *phaseoli* carrying *avrRxv*. Marker-exchange inactivation of *avrRxv* with the omega factor in *X. campestris* pv. *vesicatoria* 75-3 resulted in a change in the HR phenotype observed on bean cv. Sprite from dark to light necrosis, but not on cv. Bush Blue Lake, which gave the light necrotic HR seen with the parent strain. Inactivation of *avrRxv* did not result in extension of the host range of strain 75-3 to bean, nor to soybean, cowpea or cotton. Transfer of *avrRxv* to a range of other pathovars, including *X. campestris* pv. *glycines* on soybean, *X. campestris* pv. *vignicola* on cowpea, *X. campestris* pv. *alfalfae* on alfalfa and *X. campestris* pv. *malvacearum* on cotton conferred an ability to induce HR on their normal hosts. In *X. campestris* pv. *holcicola* on maize, no disease symptoms were observed, while no effects on pathogenicity were seen for *X. campestris* pv. *vitians* on lettuce, *X. campestris* pv. *campestris* on cabbage and *X. campestris* pv. *vesicatoria* on pepper. The latter observation is consistent with failure of the *avrRxv* gene to express in these strains or, alternatively, the absence of a functional homologue for the *Rxv* resistance gene in these hosts. The *avrRxv* gene appeared to be chromosomally located and restricted to *X. campestris* pv. *vesicatoria*, failing to hybridize with genomic DNA from other pathovars. It was noted that neither *avrBs1* nor *avrBs3* caused an HR in bean when carried by *X. campestris* pv. *phaseoli* (Whalen *et al.*, 1988).

Resistance to XcvT in tomato is rare. The subsequent discovery of a resistant cultivar of tomato to strain 75-3 and the availability of the race 2 strain 89-1 that caused disease on the same cv. Hawaii 7998, permitted investigation of the host resistance matching *avrRxv* in tomato. Examination of 587 F₂ progeny from crosses of two different susceptible tomato cultivars with Hawaii 7998 gave ratios of resistant:intermediate:susceptible progeny of 1:7:8, consistent with a requirement for two additive genes that must both be present in the homozygous dominant state for the expression of full resistance. However, it is possible that two identical resistance genes are located in different regions of the tomato genome. Certainly, F₁ progeny were consistently intermediate in their reaction type. Resistance in tomato which matches *avrRxv* was temperature sensitive in its expression, adding to the complexity of the analysis. Thus the mechanisms of resistance in bean and tomato appear to be controlled in fundamentally different ways for bacteria carrying this avirulence gene (Whalen *et al.*, 1993), an observation consistent with the findings of Fillingham *et al.* (1992) and Dangl *et al.* (1992) with respect to *avrPpiA1* from *P. syringae* pv. *pisi*.

Dosage of resistance genes in bean and tomato seems to be important, but the control of resistance is clearly different in the two hosts. This evidence

is at variance with the concept that host and non-host levels of interaction are fundamentally the same (Whalen *et al.*, 1993).

The *avrBs3* family of avirulence genes

The prototype of what has now come to be recognized as a family of genes, *avrBs3*, was located on a 45 kb plasmid designated pXV11 in *X. campestris* pv. *vesicatoria* race 1 isolate 71-21 (Minsavage *et al.*, 1990). Loss of *avrBs3* activity correlated with the loss of the plasmid. The gene hybridized to sequences in *X. campestris* pvs *alfalfae*, *campestris*, *carotae*, *glycines*, *malvacearum* and *phaseoli* (Bonas *et al.*, 1989), although subsequent probing with antibody that reacted mainly with the repeated regions detected AvrBs3 protein only in pvs *alfalfae*, *glycines*, *malvacearum*, *oryzae* and *phaseoli*. All of the latter pathovars induced HR on pepper, but the reaction was qualitatively distinct from that seen with the interaction between *avrBs3* and *Bs3* (Knoop *et al.*, 1991). DNA sequence analysis of 4363 bp (overall G + C content 65%) revealed some unique features about *avrBs3*. Two ORFs were identified: ORF1 which was later shown to correspond to *avrBs3* activity, and ORF2 on the opposing DNA strand. Within the gene, a central region comprised 17.5 direct repeats of 102 bp that coded for 34 amino acids per repeat. The repeats were 91–100% homologous with changes at between three and five amino acid positions per variant (Bonas *et al.*, 1989). The gene was expressed constitutively and independently of *hrp* genes, although it did require the presence of a functional *hrp* cluster to elicit race-specific HR *in planta*. Expression in *E. coli* resulted in the production of a 122 kDa protein that was localized intracellularly in the soluble fraction (Knoop *et al.*, 1991).

Given the structural features of *avrBs3*, it was of interest to determine whether the repeated region played some defining role in the specificity shown by the gene. A series of deletion variants were made in the repeated region that involved variable numbers and locations of repeat elements. After transfer to a race 2 recipient strain, these were tested for activity in two pepper lines: ECW30R which carried *Bs3*, and ECW which did not. From 1 to 17 repeats were deleted and the resulting variants were classed into four groups according to their reactions on the two pepper lines. Group I with two variants of 3 deleted repeats gave reactions identical to *avrBs3*. Group II with two variants of 4 deleted repeats acquired a novel avirulence phenotype that resulted in the induction of an HR on ECW, but showed loss of *avrBs3* activity toward ECW30R. Group III with three variants of 6, 10 and 12 deleted repeats gave resistant reactions on both pepper lines. Group IV, with variants ranging from 1 to 17 deleted repeats, gave susceptible reactions on both pepper lines. When tested on tomato, which does not interact with *avrBs3* and is susceptible to the race 2 recipient, all group III and those group IV variants having from 6 to 15 deleted repeats gave resistant reactions (Herbers *et al.*, 1992).

Since a number of the deletion derivatives were the same size, but belonged to different reaction groups, it is not the length of the repeated region that is critical for the determination of specificity. The position of the (deleted) repeat in the region together with the type of repeat unit present or absent seems to be the factor which determines the distinctive function of the allele. In this way changes in the internal region of the protein determine its avirulence function. Investigation of the pepper host showed that the group II derivative *avrBs3 Δ rep-16* matched a gene showing a 3:1 segregation in a cross of ECW $\times$ ECW30R, which could indicate that the recessive allele *bs3/bs3* in ECW may be behaving as a dominant resistance gene matching *avrBs3 Δ rep-16*. In addition, most of the *avrBs3 Δ rep* derivatives gave a resistant reaction in tomato, suggesting the detection of unknown resistance genes in tomato (Herbers *et al.*, 1992).

Using DNA homology to *avrBs3*, a new avirulence gene, designated *avrBs3-2*, was isolated from *X. campestris* pv. *vesicatoria* race 1 strain 82-8. This gene was located on plasmid pXV12 and conferred avirulence toward tomato cv. Bonny Best. The two genes, *avrBs3* and *avrBs3-2* are almost identical, both constitutively expressing a 122 kDa protein. The previously identified truncated gene *avrBsP* (Canteros *et al.*, 1991) is identical to *avrBs3-2* over their corresponding regions, which extend over 1.7 kb from the 5'-terminal ends of their sequences. Derivatives of *avrBs3-2* lacking the C-terminal region and part of the repetitive region were still able to elicit an HR in tomato; this accords with the situation for *avrBsP*, but is in contrast to *avrBs3* (Bonas *et al.*, 1993).

Of the 34 amino acids in each repeat unit, a limited number of positions show variation. In comparing the specificities of *avrBs3* and *avrBs3-2*, Bonas *et al.* (1993) focused on those changes occurring at positions 12 and 13. No clear conclusion could be drawn from the comparison. However, most new tomato-specific *avrBs3* alleles were small, having 11.5 or fewer repeats. None of the other *avrBs3* alleles that were analysed contained the particular repeat motif designated D, three times in tandem, except for the original *avrBs3* and two *avrBs3 Δ rep* alleles that were active on pepper (Bonas *et al.*, 1993).

All sequenced members of the *avrBs3* family, including *avrBs3*, *avrBs3-2* and *pthA* (Swarup *et al.*, 1992; see below) are flanked by 62 bp inverted repeats (IR) and homology is confined within the boundaries of these IR (Bonas *et al.*, 1993; Yang and Gabriel, 1995b).

Xanthomonas oryzae pv. oryzae

The avirulence gene *avrBs3* was used to detect cosmid clones by hybridization from a race 2 gene library of the rice blight pathogen, *X. oryzae* pv. *oryzae*. Three genes, *avrxa5*, *avrXa7* and *avrXa10*, which match rice resistance genes, *xa-5*, *Xa-7* and *Xa-10*, respectively, were detected on cosmid clones and all three genes constitute a family of genes involved in the induction of

gene-specific resistance in rice. The sequence of *avrXa-10* was very similar to *avrBs3* (Hopkins *et al.*, 1992).

Xanthomonas campestris* pv. *malvacearum

Clearly one of the most complex systems investigated to date is that studied by Dean Gabriel and co-workers involving the cotton pathogen, *X. campestris* pv. *malvacearum*. A single gene, designated *avrBn*, was recorded by Gabriel *et al.* (1994) as being an avirulence gene established by the work of Gabriel *et al.* (1986). Attempts to confirm the remaining avirulence genes described by the latter work indicated a loss of activity in the clones and a new gene library was constructed of the strain XcmH. Screening of this library in a virulent African isolate, strain XcmN, resulted in the isolation of six genes, designated *avrB4*, *avrb6*, *avrb7*, *avrBIn*, *avrB101* and *avrB102*. The genes were shown to be present on a 90 kb plasmid (pXcmH) in strain XcmH and were present in most American isolates (De Feyter and Gabriel, 1991; De Feyter *et al.*, 1993). None of the genes appeared to give a simple gene-for-gene interaction with a single resistance gene in cotton. However, *avrB4* interacts with each of two resistance genes, *B1* and *B4*, whereas resistance gene *B1* matches *X. campestris* pv. *malvacearum* strains carrying any one of *avrB4*, *avrb6* and *avrB102*. De Feyter *et al.* (1993) concluded that some cotton R genes including *B1*, *B2* and *BIn3*, react with multiple avirulence genes, while others do not, such as *B4*, *b6*, *b7* and *BIn*.

All six plasmid-borne avirulence genes described above, together with four chromosomal genes including *avrBn* (Gabriel *et al.*, 1986; Yang *et al.*, 1996), belong to a family which shows a high degree of homology to *avrBs3* and differ most obviously in the multiplicity of the tandem repeats in their central regions. These vary from 13.5 in *avrb6* to 22.5 in *avrB101* (D.W. Gabriel, University of Florida, 1996, personal communication); only *avrB4* and *avrb7* have the same number of repeats at 19. This agrees with the observations of Herbers *et al.* (1992) that both the number and position of particular variant repeats in the sequence may be critical for the specificity conferred by the particular allele. Only the *avrb6* gene appears to have been sequenced (De Feyter *et al.*, 1993).

When avirulence gene, *avrb6*, was disrupted in *X. campestris* pv. *malvacearum* strain XcmH by marker exchange, loss of avirulence toward incompatible cotton lines carrying the resistance gene *b6* and loss of virulence on susceptible cotton Acala-44 lines were observed. Growth *in planta* was not affected and similar disruption of genes *avrb7* and *avrBIn* caused loss of avirulence function but not virulence toward susceptible lines. Misting of the leaves resulted in secondary lesions forming around the main lesion in wild-type XcmH, but very few in the marker-exchanged mutant. These results, together with observations of pathogen numbers at the leaf surface, were consistent with the product of the *avrb6* gene having a role in pathogen dispersal on the cotton leaf surface (Yang *et al.*, 1994).

***pthA*: a relative of the *avrBs3* family**

X. citri is a pathogen of citrus, causing Asiatic canker, involving an induction of hyperplasia on hosts such as grapefruit. *X. campestris* pv. *citrumelo* causes citrus bacterial spot and is an opportunistic pathogen. From a gene library of *X. citri*, a gene was isolated and designated *pthA*, which conferred the ability to induce cankers in *X. campestris* pv. *citrumelo* on grapefruit. The same cosmid clone bearing *pthA* when introduced into *X. campestris* pv. *alfalfae* or *X. campestris* pv. *cyamopsidis*, both of which are weakly pathogenic on citrus, conferred the ability to induce cankers and avirulence toward their normal host plants (alfalfa and guar, respectively). Introduction of the clone into the more distantly related *X. phaseoli* and *X. campestris* pv. *malvacearum* did not affect their interactions as non-pathogens of citrus (Swarup *et al.*, 1991). However, it did result in avirulence toward their respective hosts, bean and cotton. In *X. campestris* pv. *malvacearum* on cotton, the avirulence was cultivar-specific (Swarup *et al.*, 1992). Introduction of *pthA* in *X. campestris* pv. *citrumelo* changed water-soaking on bean to an HR, consistent with an avirulence gene function for *pthA*. Marker-exchange mutagenesis of *pthA* in *X. citri* led to complete loss of virulence in terms of symptoms and *in planta* growth and loss of HR toward non-hosts (i.e. it resulted in a Hrp⁻ phenotype). Introduction of the cloned gene could restore *hrp* functions, but not growth *in planta* (Swarup *et al.*, 1991). It appeared that the non-host HR was not responsible for the 'resistance' of bean to *X. citri* (Swarup *et al.*, 1992).

Yang and Gabriel (1995b) recently used an *nptI-sac* cartridge to monitor intragenic recombination between homologous repeats of *pthA* in both *Xanthomonas* spp. and *E. coli*. New genes with novel specificities and altered pathogenicity and/or avirulence phenotypes were created. Clearly the tandemly repeated motif in these genes provides a genetic basis for the generation of multiple different avirulence specificities from a single host-specific pathogenicity gene (Yang and Gabriel, 1995b).

Comparisons of the genes *pthA* and *avrb6* showed them to be 98.4% identical in their peptide sequences and yet they determine three different phenotypes, namely cankers on citrus, water-soaking on cotton and HR on many hosts. Both *avrb6* and *pthA* enhance dispersal of the pathogen at the host plant surface; *avrb6* by release of pathogen to cotton leaf surface and *pthA* by induction of tissue hyperplasia and consequent surface rupture in citrus (Swarup *et al.*, 1991; Yang *et al.*, 1994). These releases of the pathogen to the host surface were host-specific. Recent mutational studies appear to indicate that mutation of seven avirulence genes, including *avrb6*, results in complete loss of the ability to cause symptoms in cotton. These avirulence genes appear to act additively in a quantitative fashion as pathogenicity determinants with indications that some of these functions are redundant. There was no evidence that any of the avirulence/*pth* genes affected bacterial growth *in planta*, being

merely involved in water-soaking symptoms and release of bacteria to the leaf surface (Yang *et al.*, 1996). This contrasts with the reductions in growth in *planta* associated with mutations in *avrBs2* (Kearney and Staskawicz, 1990), *pthA* in *X. citri* (Swarup *et al.*, 1991) and *avrA* and *avrE* in *P. syringae* pv. *tomato* (Lorang *et al.*, 1994).

By use of appropriate restriction endonuclease sites flanking the repeated regions of the genes *pthA* and *avrB6*, chimeric genes were constructed consisting of the 5' and 3' ends of one gene and the central repeat region from the other (i.e. ends of *pthA* and central repeats of *avrB6*, and ends of *avrB6*, with the central region of *pthA*). These constructs were introduced into appropriate recipients; *X. citri* mutant plus ends of *avrB6*/centre of *pthA* restored virulence on citrus and ends of *pthA*/centre of *avrB6* enhanced the ability of *X. campestris* pv. *malvacearum* to be pathogenic on cotton. Thus, the phenotypes conferred by these constructs were determined by the repeated regions; regions outside of the repeats were functionally interchangeable (Yang *et al.*, 1994).

Each of the seven genes, *pthA*, *avrB4*, *avrB6*, *avrB7*, *avrBln*, *avrB101* and *avrB102*, belonging to the *avrBs3* family exhibited a unique avirulence specificity in Xcm1003 on cotton resistance lines differing by single resistance genes. Specificity was always associated with the repeats. In certain cases the natural promoters appeared weak and when replaced by the *E. coli lacZ* promoter, avirulence activity was enhanced considerably (Yang *et al.*, 1994).

It was shown that *pthA* (Kingsley *et al.*, 1993) and *avrBs3* (Knoop *et al.*, 1991) require function of the *hrp* genes, specifying a type III signal peptide-independent secretion system for canker formation and avirulence, respectively. Thus, it seems likely that the proteins PthA and AvrBs3 are exported in spite of the reported failures to detect this in the case of the latter (Brown *et al.*, 1993). It is possible that the proteins are either modified or protected by chaperons or are exported at levels below the threshold of detection (Yang and Gabriel, 1995a). By examining the predicted peptides of all the sequenced members (*pthA*, *avrB6*, *avrBs3*, *avrXa10*) of the *avrBs3* family, Yang and Gabriel (1995a) detected heptad repeats similar to leucine zippers which may serve as sites for protein:protein binding or direct interaction with DNA, and three putative nuclear localization signals (NLS) in the C-terminal regions. NLS must be on the surface of the protein to function, but can be located throughout the sequence.

Translational fusions of the C-terminal regions with a *gus*-reporter gene were introduced into onion epidermal cells by microprojectile bombardment to determine whether the putative NLS might function to direct the proteins to the plant cell nucleus. Histochemical detection located *gus* activity in nuclei in three independent transformation tests. These results demonstrated that *pthA* and *avrB6* encode functional NLS, but it is not known if these are essential for the plant reaction phenotypes observed in citrus and cotton, respectively (Yang and Gabriel, 1995a). It is worth noting that for most of the *avrBs3* family, the C-terminal region of the gene is required for function (Swarup *et al.*, 1991,

1992; Hopkins *et al.*, 1992; Bonas *et al.*, 1993; De Feyter *et al.*, 1993). In the case of *avrBs3-2*, up to two-thirds of the C-terminal part of the gene can be deleted (including the NLS) without loss of avirulence function when introduced into *X. campestris* pv. *vesicatoria* on tomato (Bonas *et al.*, 1993).

Fenselau *et al.* (1992) proposed that *X. campestris* pv. *vesicatoria* secretes AvrBs3 protein directly into the intercellular spaces within the plant mesophyll. Yang and Gabriel (1995a) further proposed that AvrBs3-like gene products could be taken up into the plant cells by receptor-mediated endocytosis (Horn *et al.*, 1989) and translocated to the nucleus of the cell. Avirulence proteins or protein complexes may act on nuclear transcriptional factors, leading to different physiological outcomes such as hyperplasia of citrus, water-soaking of cotton or the HR (programmed cell death) (Yang and Gabriel, 1995a).

The Role of *hrp* Genes

Isolation and DNA sequence analysis of *hrp* genes from *P. syringae* pv. *phaseolicola*, *P. syringae* pv. *syringae*, *X. campestris* pv. *vesicatoria*, *B. solanacearum* and *E. amylovora* revealed that they were clustered and some at least showed homology to genes involved in a protein secretion pathway of virulence factors called Yops found in the animal pathogen, *Yersinia*. Considerable similarity exists between those of *B. solanacearum* and *X. campestris* (Fenselau *et al.*, 1992; Gough *et al.*, 1992). In *E. amylovora*, *E. chrysanthemi* and *P. syringae*, *hrp* genes have been shown to control the production and secretion of glycine-rich protein elicitors of the HR, termed harpins, which are essential for pathogenicity (He *et al.*, 1993; Wei and Beer, 1993; Bauer *et al.*, 1995). A further glycine-rich protein elicitor, called PopA, which elicits HR in leaves of the non-host tobacco, was isolated from *B. solanacearum* (Arlat *et al.*, 1994). However, the phenotype of the *popA* gene more closely resembles an avirulence gene, in that mutants do not lose the ability to cause an HR in tobacco and cause disease in tomato. This suggests that such glycine-rich proteins may have quite different roles among Gram-negative plant pathogens (Bauer *et al.*, 1995).

The *hrp* genes are widespread and conserved between Gram-negative phytopathogenic bacteria (Willis *et al.*, 1991; Bonas, 1994). Clusters are located on the chromosome in *P. syringae* (Rahme *et al.*, 1991) and *X. campestris* pv. *vesicatoria* or on a megaplasmid in *B. solanacearum* (Boucher *et al.*, 1986, 1988). The conditions for induction of *hrp* genes vary in terms of the specific components that have been identified for different genera (Lorang and Keen, 1995). Thus, Rahme *et al.* (1992) showed in *P. syringae* pv. *phaseolicola* that low osmolarity, pH and carbon source were important. In *X. campestris* pv. *vesicatoria*, Schulte and Bonas (1992a) showed that it was phosphate and sodium chloride concentrations and the presence of sulphur-containing amino acids. And, in *Erwinia amylovora*, Wei *et al.* (1992b) showed that ammonium

ions, nicotinic acid, complex nitrogen sources, temperature and pH were important. All systems shared a common response to induction with sucrose, while showing variable responses with other carbon sources (Huynh *et al.*, 1989; Arlat *et al.*, 1991, 1992; Schulte and Bonas, 1992a; Wei *et al.*, 1992b; Xiao *et al.*, 1992).

Control of *hrp* gene expression in *P. syringae*

hrpS was first described by Grimm and Panopoulos (1989) and its product is a 34 kDa protein related to sigma-54 bacterial enhancer binding proteins. The genes *hrpR* and *hrpS* in *P. syringae* pv. *syringae* are very similar in their structural organization to *hrpRS* in *P. syringae* pv. *phaseolicola* but differ functionally. Xiao *et al.* (1994) postulated that in *P. syringae* pv. *syringae*, *hrpR* constitutes a transcriptional activator of the response regulator *hrpS*, whereas in *P. syringae* pv. *phaseolicola*, HrpR and HrpS might function as a dimer. Both *P. syringae* pv. *phaseolicola* genes may be orphan regulators without sensors from ancestral two-component systems. There are other similarities to the *alg* and *xyl* operons from *Pseudomonas* and a rhamnose operon in *E. coli* (Grimm *et al.*, 1995). These genes activate *hrpL*, the product of which induces expression of HrpL-responsive genes (Xiao *et al.*, 1994). Since many avirulence and *hrp* genes possess a so-called 'hrp-box' sequence upstream of their open reading frames, they are nutritionally regulated by the *hrpRS* and *hrpL* system. The functional significance of this arrangement is the subject of intense investigation at this time and is addressed below.

Control of *hrp* gene expression in *Xanthomonas* spp. and *B. solanacearum*

Expression of *hrp* genes is suppressed in rich media but induced in minimal media or *in planta* (Schulte and Bonas, 1992b). The *X. campestris* pv. *vesicatoria* promoter of the *hrpB* operon is a plant-inducible promoter (PIP) box, 44 bp upstream from the transcription start site (Fenselau and Bonas, 1995); it is not related to sigma-54 or sigma-70 transcription binding sites or *hrp* box. Although putative *hrp* box sequences have been suggested for *avrRxv*, *avrBs3*, *avrBsP*, *avrXa10* and *avrXca*, these are unrelated to the PIP box and do not appear to be important in *Xanthomonas* (U. Bonas, Gif-sur-Yvette, 1996, personal communication). Identical PIP boxes are also found upstream of the *X. campestris* pv. *vesicatoria* *hrpC*, *hrpD* and *hrpF* operons, the avirulence gene, *avrRxv* (Whalen *et al.*, 1993), and *hrp* transcription units II (= *hrpB*), III (= *hrpC*) and IV (= *hrpD*) of *B. solanacearum*. The relationship of the PIP

boxes to the transcription start sites of these genes remains unclear (U. Bonas, Gif-sur-Yvette, unpublished, cited in Fenselau and Bonas, 1995).

The HrpB3 protein from *X. campestris* pv. *vesicatoria* is a putative lipoprotein localized in the outer membrane and carrying a signal peptide sequence at its N-terminus. This protein is conserved across several genera, including animal pathogens, as HrpI (*B. solanacearum*: Van Gijsegem *et al.*, 1995), HrpC (*P. syringae* pv. *syringae*: Preston *et al.*, 1995), YscJ (*Yersinia* spp.: Lidell and Hutcheson, 1994), MxiJ (*Shigella flexneri*: Allaoui *et al.*, 1992), PrgK (*Salmonella typhimurium*: Pegues *et al.*, 1995) and NolT (*Rhizobium fredii* – a symbiont of soybean; Meinhardt *et al.*, 1993). HrpB6 (a putative ATPase) and HrpB8 show similarities to type III protein secretion pathway components (Fenselau and Bonas, 1995).

At the left end of the *hrp* cluster is *hrpA*, which encodes a single 64 kDa protein, HrpA1, belonging to the PulD superfamily involved in type II and type III protein secretion. The inducible promoter is unrelated to known promoter elements and expression of *hrpA* was found to be independent of the *hrpX* regulatory gene. HrpA1, which is located in the outer membrane of *X. campestris* pv. *vesicatoria*, most likely forms multimers (Wengelnik *et al.*, 1996).

The relationship between hrp and avirulence genes

Using the *avrB* gene from *P. syringae* pv. *glycinea*, Huynh *et al.* (1989) were able to demonstrate by manipulation of carbon sources in defined (minimal) media that expression of this gene was induced in the presence of sugars such as fructose, sucrose and mannitol and repressed by substrates such as citrate, succinate and peptone. Growth substrates entering the Entner–Doudoroff pathway of carbohydrate catabolism at the pyruvate step or earlier appeared not to repress *avrB*. The genes now known to be *hrpL*, *hrpR* and *hrpS* were shown to be required for the transcription of the *avrB* gene in culture and also *in planta*. The induction was associated with a rapid turnover of elicitor activity of some 10 min, and expression was not under the control of the soybean resistance gene *RPG1* (Huynh *et al.*, 1989). However, comparison of the upstream region of *avrRpt2* with that of nine other *P. syringae* avirulence genes identified a highly conserved sequence located 6 to 8 nucleotides upstream of the transcriptional start site (Innes *et al.*, 1993b). Only part of this sequence was originally called an *hrp* box; this part was first noted by Jenner *et al.* (1991) and Salmeron and Staskawicz (1993).

In an examination of promoter function in the upstream region of the *avrD* gene from *P. syringae* pv. *tomato*, Shen and Keen (1993) found that promoter activity was repressed by high concentrations of nitrogenous compounds and pH above 6.5. They used primer extension analysis to show that the transcription initiation site was 41 nt upstream of the translational start site when cells were grown in soybean leaves. At 14 nt upstream of the initiation site was a

sigma-54 promoter consensus GG-10 nt-GC and deletions 3' of this site abolished promoter activity. When introduced into a mutant of *P. syringae* pv. *phaseolicola* deficient in the *ntrA*-specified (sigma-54) cofactor, there was little expression of a *gus* fusion with the *avrD* promoter, confirming the requirement for this activity, together with that of the *hrpS* and *hrpL* genes (Shen and Keen, 1993).

There are common features of *avrE* with two genes encoding harpins: *hrpZ* of *P. syringae* pv. *syringae* and *hrpN* from *E. amylovora*. Mutation of *avrE* in *P. syringae* pv. *tomato* and *hrpZ* in *P. syringae* pv. *syringae* reduced but did not eliminate virulence (He *et al.*, 1993). However, *avrE* still elicited an HR on non-hosts whereas *hrpZ* did not. Unlike *hrpN* (Wei *et al.*, 1992a), *avrE* may be plant species-specific since it does not cause *P. syringae* pvs *lachrymans*, *tobacco*, *phaseolicola*, *syringae* or *pisi* to elicit the HR on 'their normal hosts'. Thus, *avrE* may serve as a link between *hrp* and avirulence genes (Lorang and Keen, 1995).

LemA/Gac: A Two Component Sensor/Regulator

Although not strictly either an *hrp* or an avirulence gene, it is appropriate to mention here a further example of a two-component bacterial sensor/regulator system that may be a global regulator influencing the interaction between some host/pathogen combinations. Willis *et al.* (1990) investigated the *P. syringae* pv. *syringae* strain 728a, which is a pathogen of bean causing brown spot disease. A mutation, designated *lemA*, results in the absence of lesions on bean and loss of protease and syringomycin production, but remains capable of inducing an HR in tobacco. No role has yet been assigned to protease production or syringomycin in lesion formation and mutants obtained indicate that their production is not sufficient for the appearance of symptoms in bean (Willis *et al.*, 1994). Cloning and sequencing revealed that LemA has a predicted molecular mass of 113 kDa and is a member of a two-component family of bacterial positive transcriptional regulators (Hrabak and Willis, 1992). Hybridization studies have shown *lemA* to be ubiquitous in *P. syringae* and homologues to be present in both plant and animal pathogens and saprophytes including *X. campestris*, *B. solanacearum*, *Aeromonas hydrophila*, *A. salmonicida*, *P. fluorescens* and *P. aeruginosa* (Rich *et al.*, 1992; Willis *et al.*, 1994).

A *lemA* homologue was cloned from the halo-blight pathogen *P. syringae* pv. *phaseolicola* strain NPS3121 and shown to restore the full phenotype of a *lemA* mutant of *P. syringae* pv. *syringae*. However, marker-exchange mutagenesis of the *P. syringae* pv. *phaseolicola* *lemA* gene did not abolish the production of halo-blight symptoms or toxin production in bean (Rich *et al.*, 1992). The introduction of a *lemA* mutation into the oat pathogen *P. syringae* pv. *coronafaciens* resulted in loss of tabtoxin production but did not affect lesion formation (Barta *et al.*, 1992).

Using a procedure that involved repeated growth of *P. syringae* pv. *syringae* strain 728a to stationary phase over five cycles, spontaneous mutants were obtained that mimicked *lemA* mutants but were genetically distinct from it. Independently working with *P. fluorescens*, Laville *et al.* (1992) had used a similar procedure to obtain a global regulator, designated *gacA*, which controlled the production of several extracellular antibiotics and cyanide. Hybridization studies confirmed that the spontaneous (and UV-sensitive) mutants, which mimicked *lemA*, were complemented by the *gacA*-containing clone from *P. fluorescens* (Rich *et al.*, 1994).

The *gacA* gene clearly encodes a response regulator for the unlinked *lemA* sensor kinase. GacA is a member of the FixJ family of bacterial response regulators and there was 92% identity of the GacA proteins from *P. syringae* and *P. fluorescens*. The UV-sensitivity of the spontaneous *gacA* mutants was due to polar effects of the mutations on a *uvrC* analogue, located downstream of *gacA* in *P. syringae* pv. *syringae*. One interesting aspect of the *gacA* mutants was that while in *P. fluorescens* they were due to deletions of the region of the genome containing *gacA*, there was no evidence of deletions in the formation of the *gacA* mutants in *P. syringae* pv. *syringae*. Given the procedure used to isolate the *gacA* mutants, it is possible that *lemA/gacA* may modulate cellular functions that are detrimental to survival during stationary phase in laboratory media (Rich *et al.*, 1994).

Concluding Remarks

Gabriel (1989) argues that it is the 'positive' pathogenicity functions that determine host range at pathovar/plant species level and that avirulence gene functions at this level of interaction are gratuitous or incidental. Thus there are *hsn* genes for nodulation specificity in *Rhizobium* spp. and *hsv* genes for species-specific virulence in *Xanthomonas* spp. (Waney *et al.*, 1991), *P. syringae* pv. *tabaci* (Salch and Shaw, 1988) and *B. solanacearum* (Ma *et al.*, 1988), although few of the latter appear to have been pursued subsequently. Thus, the HR induced in bean by *X. citri* is not responsible for the limitation of its host range on bean (Swarup *et al.*, 1992).

Given the structural features which set the AvrBs3 family of avirulence gene products apart from the remainder, it seems likely that there are fundamental differences in the mechanisms by which the *avrBs3*-like genes interact with the plant host compared with the ones from *P. syringae*; although, even these may not all function in a similar way (compare *avrD* and the rest). Recent suggestions that the AvrBs3 peptides may find their way to the nucleus of the plant cell might indicate some direct role in control of the host plant response through differential gene expression.

The role of *hrp* genes in relation to the *avrBs3*-like genes in particular, appears to be much more exciting than first supposed, with the discovery

of NLS on some the peptides from avirulence genes from *X. campestris* pv. *malvacearum*. The dependence of many avirulence genes on components of the Hrp secretion system raises the possibility that it functions to translocate the products of avirulence genes to sites for uptake by plant cells.

Amid all of the speculation, it is tempting to conclude that enough is known from over 30 avirulence genes already characterized about the bacterial contribution to host/pathogen interactions. As a consequence, the swing of resources towards the investigation of the plant host will certainly ensure that rapid progress towards the goal of engineered and durable resistance is effectively pursued. However, many important questions remain unanswered about avirulence genes. How do their gene products interact with resistance gene products? What other roles do they have? Are they fitness determinants for the pathogen? Is there any significance in their genomic location, either chromosomal or plasmid-borne, within isolates? How mobile are avirulence genes in field situations? Given the relatively low average G + C values for *Pseudomonas* avirulence genes, where did they originate? Are low G + C values maintained for some reason within pseudomonads, which otherwise have high overall G + C genomes? One thing is certain, we are still some way from a full understanding of these fascinating genes.

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Molecular Characterization of Fungal Avirulence

17

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The host range of a fungal pathogen is in many cases restricted to a single plant species. The molecular mechanisms underlying the successful colonization of a particular host by a parasite or the induction of resistance in non-hosts, the so-called host species specificity, remain poorly understood. In contrast, the genetic basis of cultivar specificity has been the object of extensive research. More than 50 years ago, Harold Flor demonstrated that the outcome of an interaction between different races of the rust fungus, *Melampsora lini*, and cultivars of the host species, flax, is governed by a gene-for-gene relationship (Flor, 1942, 1971). An incompatible interaction (resulting in plant resistance as opposed to a compatible interaction where disease occurs) ensues if the host cultivar contains a usually dominant resistance gene that corresponds to an avirulence gene carried by the pathogen. Avirulence genes are envisioned to encode race-specific elicitor molecules which interact with specific plant receptors encoded by resistance genes (Gabriel and Rolfe, 1990; Keen, 1990). Early molecular recognition results in many cases in the rapid death of a few cells at infection sites (hypersensitive response, HR) and the activation or induction of many defence-associated reactions (reviewed by Kombrink and Somssich, 1995).

The gene-for-gene hypothesis has now been extended to interactions involving plants and other classes of pathogens such as viruses, bacteria, nematodes and insects as well as to other plant/fungus interactions including lettuce/*Bremia lactucae*, tobacco/*Phytophthora parasitica*, grass species/*Magnaporthe grisea*, tomato/*Cladosporium fulvum* and barley/*Rhynchosporium secalis* (Crute, 1985; de Wit, 1995). Avirulence genes from *B. lactucae* and *M. lini* have been characterized by classical genetic analyses and will be cloned in the near

future. The *parA1* gene product from *P. parasitica* may be considered as a species-specific elicitor (Kamoun *et al.*, 1994). However, further genetic analysis is necessary to confirm that *parA1* is a genuine avirulence gene. In this chapter, we will therefore focus on avirulence genes from *C. fulvum*, *R. secalis* and *M. grisea*. In addition, recent data provided evidence that two *M. grisea* genes controlling host species specificity appear to have characteristics analogous to cultivar-specific avirulence genes and will be included in this chapter.

***Cladosporium fulvum* and Tomato Interaction**

The imperfect biotrophic fungus, *Cladosporium fulvum*, is the causal agent of leaf mould of tomato, which is the only host it can infect. After penetration via the stomata, mycelia colonize the intercellular spaces between mesophyll cells and stay confined to the apoplast during the main part of the life cycle (Lazarovits and Higgins, 1976; de Wit, 1977). At early stages of infection, the fungus does not cause any visible damage to the leaf tissue and probably obtains nutrients from the apoplast since no specialized feeding structures such as haustoria are formed. In contrast, in an incompatible interaction, fungal growth is restricted and necrotic lesions rapidly occur on resistant tomato leaves.

In tomato, at least 11 genes conferring resistance to *C. fulvum* have been described. Some of them, *Cf-2*, *Cf-3*, *Cf-4*, *Cf-5* and *Cf-9*, have been introgressed into cultivar MoneyMaker which contains no known resistance gene (referred to as *Cf-0*) to generate near-isogenic lines. The differential response (resistance or susceptibility) observed between different races of *C. fulvum* and these near-isogenic tomato lines suggested that the *C. fulvum*/tomato interaction is governed by a gene-for-gene relationship. However, the presence of single avirulence genes could not be demonstrated genetically as *C. fulvum* does not have a known sexual stage. Since fungal growth is restricted to the apoplast, race-specific elicitors have been searched for in intercellular fluids of tomato leaves colonized by the fungus (de Wit and Spikman, 1982; de Wit *et al.*, 1984, 1985). Two peptides were purified, AVR4 and AVR9, which specifically induce HR in tomato plants carrying the complementary resistance gene, *Cf-4* and *Cf-9* respectively (Schottens-Toma and de Wit, 1988; Joosten *et al.*, 1994). Based on the elicitor amino acid sequences, degenerated oligonucleotides were designed and used to isolate cDNA and genomic clones (van Kan *et al.*, 1991; van den Ackerveken *et al.*, 1992; Joosten *et al.*, 1994). *Avr4* and *Avr9* thus became the first fungal avirulence genes to be cloned.

The avirulence gene Avr9

The *Avr9* gene contains a short intron of 59 bp and encodes a preproprotein of 63 amino acids including the sequence of the mature elicitor of 28 amino

acids at the C-terminal end (van Kan *et al.*, 1991; van den Ackerveken *et al.*, 1992). The native AVR9 protein contains a putative signal peptide of 23 amino acids (van Kan *et al.*, 1991). However, the resulting proprotein of 40 amino acids has never been detected experimentally as it is processed rapidly by fungal and plant proteases to intermediate forms of 32 to 34 amino acids and to the 28 amino acid elicitor peptide (van den Ackerveken *et al.*, 1993).

Southern analysis showed that all races that are avirulent on Cf-9 tomato plants contain a single copy of *Avr9* whereas all virulent races lack the gene (van Kan *et al.*, 1991). Transformation of a virulent race with *Avr9* conferred to the resulting strain avirulence specifically on Cf-9 tomato plants (van den Ackerveken *et al.*, 1992). Furthermore, disruption of *Avr9* in an avirulent race led to virulence (Marmeisse *et al.*, 1993). These results demonstrated that *Avr9* is the only fungal genetic factor required to determine avirulence on Cf-9 tomato plants.

Avr9 is highly expressed when the fungus grows inside tomato leaves but not under optimal growth conditions in liquid culture (van Kan *et al.*, 1991). Histochemical localization of GUS activity reporting *Avr9* expression showed that the gene is strongly induced after penetration of the fungus via the stomata. Highest levels of GUS activity were detected in mycelia growing near the vascular tissues of the leaf (van den Ackerveken *et al.*, 1994). *Avr9* expression is strongly induced *in vitro* in growth medium containing low amounts of nitrogen, probably reflecting the conditions found in the apoplast (van den Ackerveken *et al.*, 1994). Limitation of other macronutrients or the addition of plant factors to the growth medium fails, however, to induce *Avr9* expression. The *Avr9* promoter contains six copies of the motif TAGATA and six additional copies of the core sequence, GATA (van den Ackerveken *et al.*, 1994). These consensus sequences have been identified as the recognition site of the *Neurospora crassa* NIT2 protein (Fu and Marzluf, 1990), which induces the expression of many genes under nitrogen-limiting conditions. Deletion of several of these motifs appears to abolish *Avr9* induction under low nitrogen conditions, suggesting that the expression of *Avr9* is mediated through a positive-acting nitrogen regulatory protein (de Wit, 1995).

Structure and putative function of the AVR9 protein

The elicitor-active 28 amino acid peptide contains six cysteines that are involved in disulphide bridges, as was revealed by electrospray mass spectrometry. Formation of disulphide bridges is required for elicitor activity since complete reduction of AVR9 abolishes HR-induction. Elucidation of the AVR9 tertiary structure by 2D-NMR yielded a compact barrel-like molecule comprising three antiparallel β -sheets connected by two loops of two and ten amino acids (Honée *et al.*, 1994; de Wit, 1995).

To determine the relative importance of specific amino acids for AVR9 elicitor activity, mutations were introduced into the *Avr9* sequence. Single amino acid exchanges showed different types of effects on AVR9 function ranging from no or limited HR-induction to necrosis occurring more rapidly than with the wild-type elicitor. Several amino acids were thus found to be important for elicitor activity, among them the carboxy-terminal histidine, which when replaced by leucine strongly reduces necrotic activity (Honée *et al.*, 1994). In addition, a synthetic peptide of 27 amino acids lacking this last amino acid is biologically inactive on Cf-9 tomato plants and poorly soluble in water, suggesting that the C-terminal end of AVR9 plays an important role in the structure, stability and/or activity of the protein (de Wit, 1995).

The intrinsic function of AVR9 during a compatible interaction is not known. The high level of *Avr9* transcript in hyphae in the vicinity of vascular bundles may indicate that AVR9 plays a role in mediating nutrient movement from the vascular tissues into the apoplast (van den Ackerveken *et al.*, 1994). Under laboratory conditions, *Avr9* appears to be dispensable since disruption of the gene did not affect growth and pathogenicity of *C. fulvum* (Marmeisse *et al.*, 1993). In nature, however, loss of *Avr9* might interfere with the fitness of the fungus as races of *C. fulvum* lacking the gene do not migrate out of the different geographic locations where they showed up (Honée *et al.*, 1994). Furthermore, the Cf-9 gene introduced into tomato breeding lines in 1979 still provides a good protection against *C. fulvum*, suggesting that if new virulent races appear they are less pathogenic (de Wit, 1995). Nevertheless, it should be mentioned that karyotype analysis of two races virulent on Cf-9 tomato lines revealed that both contain large deletions encompassing *Avr9* and linked genes which may also play a role in virulence or fitness of the fungus (Talbot *et al.*, 1991).

The avirulence gene *Avr4*

The intron-less gene *Avr4* is expressed *in planta*. However, unlike *Avr9* it is not induced under nitrogen-limiting conditions *in vitro*. *Avr4* encodes a mature preproprotein of 135 amino acids including a putative N-terminal signal peptide of 18 amino acids. The cleaved protein of 117 amino acids is processed further to an elicitor-active protein of 105 amino acids by plant or fungal proteases. The mature AVR4 protein is also cysteine-rich, containing eight of these amino acids, and shows no significant homology with other proteins in the database (Joosten *et al.*, 1994).

Southern blotting showed that all *C. fulvum* races contain an *Avr4* gene. The deduced amino acid sequences of *Avr4* alleles from all avirulent races are identical. However, single amino acid alterations were identified in the primary structure of AVR4 proteins from virulent races (Joosten *et al.*, 1994). In seven cases, a cysteine residue was found to be replaced by a tyrosine. In addition, two other amino acid exchanges (from tyrosine to histidine and from threonine

to isoleucine) were detected between the fourth and the fifth cysteine. In one case, a nucleotide deletion gave rise to a frame shift, leaving at the N-terminus 13 amino acids of the wild-type AVR4 protein. Northern blotting showed that all *Avr4* alleles are transcribed during infection. In contrast, none of the proteins encoded by the altered *Avr4* alleles were detected by western blotting of intercellular fluids, suggesting that the gene products are unstable or not secreted (Honée *et al.*, 1994; de Wit, 1995).

Transformation of a virulent race containing a mutated *Avr4* allele with a functional gene confers avirulence to the resulting strain and the ability to produce a protein inducing an HR specifically on Cf-4 tomato plants. The wild type *Avr4* gene appears, therefore, to be dominant over the mutated *Avr4* gene (Joosten *et al.*, 1994). Since the strain producing a truncated AVR4 protein is virulent, a functional *Avr4* gene is not only sufficient but also necessary to determine avirulence on Cf-4 plants (de Wit, 1995). These results show that *Avr4* fully complies with the definition of an avirulence gene.

The Cf proteins and the AVR9 receptor

The elicitor/receptor model states that the avirulence gene product interacts with a plant receptor encoded by the resistance gene. To test this hypothesis, resistance genes were isolated by either transposon tagging or positional cloning. The sequences of the resistance genes *Cf-2* and *Cf-9* have been reported (Jones *et al.*, 1994; Dixon *et al.*, 1996). The deduced Cf protein structures share the same hallmarks. They appear to be glycoproteins containing a putative secretory signal peptide, a single transmembrane domain, and a short cytoplasmic tail lacking any obvious intracellular signalling domain. The main part of the proteins is thought to be extracellular with a large domain containing leucine-rich repeats (LRRs). LRRs are involved in the recognition of small peptides or in protein/protein interaction. *Cf-9* contains 28 imperfect LRRs, whereas *Cf-2* possesses 33 perfect and five imperfect LRRs. Sequence comparison of *Cf-2* and *Cf-9* shows that the C-terminal LRRs are more conserved than those near the N-terminus (Dixon *et al.*, 1996). It is therefore possible that at least part of the LRR domains is involved in the recognition of the different AVR peptides, whereas the more conserved LRRs may interact with other proteins involved in the signal transduction pathways, leading to resistance.

The binding properties of the AVR9 peptide have been tested to determine whether the *Cf-9* gene encodes the AVR9 receptor. Binding studies using radioiodinated AVR9 peptide revealed a single class of high-affinity binding sites in plasma membranes from resistant (*Cf-9*) and susceptible (*Cf-0*) tomato plants as well as from other solanaceous plants (Kooman-Gersmann *et al.*, 1995). This result raises the question of how cultivar specificity is determined during the interaction of AVR9-producing fungal strains and *Cf-9* tomato plants. Southern and Northern blotting demonstrated the presence of *Cf-9*

homologues and a single RNA of similar size in both resistant and susceptible tomato plants, suggesting the presence of Cf-9-like proteins in both cultivars (Jones *et al.*, 1994). At least two hypotheses can be put forward to explain the cultivar specificity: (i) the structure of Cf-9-like proteins from both resistant and susceptible cultivars is similar in the region interacting with AVR9, but differs in the domain involved in signal transduction; (ii) AVR9 binds in resistant plants to a not yet identified plasma membrane protein interacting with Cf-9, the latter being only in resistant plants as a component of the signal transduction pathway leading to HR induction.

Plant defence responses induced during incompatible interactions

Different groups reported specific plant defence responses induced by avirulent races, purified race-specific elicitors or intercellular fluids containing AVR2 or AVR5 which induce an HR specifically on Cf-2 and Cf-5 tomato plants, respectively. However, no complete signal transduction pathway can be drawn thus far. In resistant plants, avirulent races induce the deposition of callose (Lazarovits and Higgins, 1976) as well as the accumulation of phytoalexins (de Wit and Flach, 1979) and of pathogenesis-related (PR) proteins including P14 (a tomato PR1a homologue; de Wit and van den Meer, 1986), chitinases and 1,3- β -glucanases (Joosten and de Wit, 1989). Transient expression of acidic and basic chitinases and 1,3- β -glucanases is also induced by AVR9 specifically in Cf-9 tomato plants (Ashfield *et al.*, 1994; Wubben *et al.*, 1996). In comparison, AVR4 stimulates a differential accumulation only of the acidic enzymes in Cf-4 tomato plants (Wubben *et al.*, 1996). These results suggested that the tomato chitinases and 1,3- β -glucanases may be involved in the degradation of hyphal walls. However, *C. fulvum* appears to be insensitive *in vitro* to these hydrolytic enzymes, leaving their role in resistance to the fungus uncertain (Joosten *et al.*, 1995).

Injection of AVR9-containing intercellular fluids into cotyledons of Cf-9 seedlings resulted in several physiological responses: elevated levels of total and oxidized glutathione; increases in lipoxygenase activity, lipid peroxidation and electrolyte leakage; and an oxidative burst (Peever and Higgins, 1989; Hammond-Kosack and Jones, 1995). In addition, ethylene production occurred followed by a significant increase in free salicylic acid coincident with the loss in membrane integrity (Hammond-Kosack and Jones, 1995). Similar responses were obtained with AVR2-containing intercellular fluids and Cf-2 tomato seedlings. However, collapse of epidermal cells, ethylene production and loss of cell viability appeared to be delayed by 4 to 7 h in Cf-2 plants (Hammond-Kosack and Jones, 1995).

Vera-Estrella *et al.* (1992) studied the effects of specific elicitors using tomato suspension cells which were shown to have retained the specificity of

intact plants. A rapid increase in active oxygen species, in extracellular peroxidase activity and in phenolic compounds was found only upon addition of AVR5-containing intercellular fluids to tomato cells carrying the resistance gene *Cf-5*. In plasma membranes, a crude AVR5 preparation induced a fourfold increase in H^+ -ATPase activity, acidification of the extracellular medium (Vera-Estrella *et al.*, 1994a) as well as redox changes (Vera-Estrella *et al.*, 1994b). Both the redox changes and ATPase stimulation appear to be mediated by G-proteins and phosphatase which are possibly activated upon interaction of AVR5 with its receptor.

***Rhynchosporium secalis* and Barley Interaction**

The imperfect fungus, *Rhynchosporium secalis*, is the causal agent of barley leaf scald. The infection cycle starts with the penetration of the leaf cuticle by the fungus, which stays confined to the subcuticular region throughout most of its life cycle. The early stages of pathogenesis are characterized by the collapse of a few epidermal cells. Subsequently, mesophyll cells underlying the affected epidermal cells collapse. As a perthotrophic fungus, *R. secalis* appears to kill host cells as a source for nutrients. At later stages of infection, the subcuticular mycelium forms a dense stroma, causes the formation of necrotic lesions and finally sporulates (Lehnackers and Knogge, 1990). In incompatible interactions, the early infection stages are similar to those found in compatible interactions. However, fungal growth is arrested after the collapse of a few epidermal cells, probably as a result of the induction of plant defence genes. An HR does not occur and no symptoms are macroscopically visible on resistant barley leaves (Lehnackers and Knogge, 1990).

Resistance of barley to *R. secalis* is governed by several major resistance genes, among them, the codominant *Rrs1* gene (Hahn *et al.*, 1993). Several fungal races that are avirulent on *Rrs1* plants but virulent on *rrs1* plants were isolated (Rohe *et al.*, 1995). This suggested that these strains possess the avirulence gene, *AvrRrs1*, matching the resistance gene, *Rrs1*, and that the interaction of *R. secalis* with barley complies with the gene-for-gene hypothesis.

The dual functions of NIP1

In the search for molecules involved in fungal virulence, three necrosis-inducing proteins were identified, referred to as NIP1, NIP2 and NIP3. These NIPs are small secreted proteins with molecular masses of less than 10 kDa found in fungal culture filtrates as well as in infected susceptible plants, their occurrence correlating with lesion development (Wevelsiep *et al.*, 1991). Toxicity of NIP1 and NIP3 appears to be mediated through a stimulation of the plant plasmalemma H^+ -ATPase in both susceptible and resistant barley plants

as well as in other mono- and dicotyledonous plants (Wevelsiep *et al.*, 1993). The mode of action of NIP2 is not known.

Resistance of barley to *R. secalis* is expressed without macroscopically detectable alterations of the plant tissues. Upon inoculation of *Rrs1* plants with an avirulent strain, the host response is characterized by a rapid and transient induction of mRNAs encoding peroxidase and PR5-like proteins. Only NIP1 was capable of inducing these mRNA species specifically in plants of the *Rrs1* genotype when the NIPs and other fractions from fungal culture filtrates and cell walls were analysed for elicitor activity (Hahn *et al.*, 1993). Furthermore, inoculation of barley plants with a mixture of spores from a virulent race and the purified NIP1 prevented this race from infecting *Rrs1* but not *rrs1* plants (Rohe *et al.*, 1995). NIP1 therefore possesses elicitor activity, in addition to its toxic activity, and was presumed to be the product of the fungal avirulence gene, *AvrRrs1*.

NIP1 is the product of the avirulence gene AvrRrs1

Based on the amino acid sequence of the purified NIP1, degenerated oligonucleotide primers were designed and used in a PCR-assisted (polymerase chain reaction) approach to isolate genomic and cDNA clones. The *nip1* gene consists of two exons separated by a 65 bp intron. The deduced amino acid sequence revealed a putative signal peptide of 22 amino acids. The mature protein contains 60 amino acids, 10 of which are cysteines (Rohe *et al.*, 1995). Preliminary data indicate that these cysteines are involved in disulphide bridges, since reduction of NIP1 abolishes elicitor activity.

Southern blotting revealed that all races lacking the *nip1* gene are virulent on *Rrs1* plants while all avirulent races possess a *nip1* homologue. The deduced amino acid sequences of the *nip1* alleles from different avirulent races fall into two groups which differ in three amino acids. Alterations involve the replacement of alanine-40 by glutamate, histidine-43 by glutamine and threonine-77 by lysine. Both types of NIP1 are elicitor-active. In contrast, elicitor activity of NIP1 from two races was abolished by single additional amino acid exchanges involving the replacement of serine-45 by proline and of glycine-67 by arginine (Rohe *et al.*, 1995). Experiments involving *in vitro* mutagenesis are in progress to determine whether these latter exchanges alone or in combination with the other amino acid alterations are responsible for the loss of elicitor activity.

The causal relationship between a *nip1* gene encoding an elicitor-active protein and avirulence was demonstrated by genetic complementation and gene disruption. Transformation of a virulent race with a functional *nip1* gene renders this strain avirulent on *Rrs1* plants showing that *nip1* is sufficient to determine avirulence on resistant plants (Rohe *et al.*, 1995). Disruption of *nip1* in a race avirulent on *Rrs1* plants but virulent on *rrs1* plants confers to that

strain the ability to induce symptoms on both cultivars. This is the final proof that *nip1* is the only fungal genetic factor that determines avirulence in combination with the *Rrs1* gene and that it is identical with the avirulence gene, *AvrRrs1*. However, the *nip1* disruption mutant was found to be less virulent on both *Rrs1* and *rrs1* barley plants than the parental strain on *rrs1* plants (Knogge, unpublished data). The virulence phenotype of this mutant strain is similar to that of wild-type races lacking the *nip1* gene, confirming that the toxic role of NIP1 in virulence is not negligible. Currently, one of the latter fungal races is being transformed with *nip1* to determine whether full virulence of the resulting strain on *rrs1* plants can be obtained.

Are both NIP1 functions mediated through the same receptor?

In compatible interactions, NIP1 is produced in high amounts by the growing fungus and functions as a virulence factor by stimulating the plasma membrane H^+ -ATPase and by inducing necrosis. In incompatible interactions, NIP1 acts at very low concentrations as an elicitor of plant defence reactions that result in the arrest of fungal growth. Three peptides spanning the complete primary sequence of the mature protein were synthesized to elucidate whether different domains of NIP1 are involved in its two functions. These peptides, alone or in all possible combinations, failed to induce the accumulation of PR5 mRNA. However, necrosis was induced in resistant and susceptible barley plants with all combinations including peptides corresponding to the central and the C-terminal part of NIP1 (Li and Knogge, unpublished data). Experiments are in progress to determine whether these peptides also stimulate the plasma membrane H^+ -ATPase.

How can a single molecule exert such contrasting functions during pathogenesis? Three hypotheses can be put forward to explain toxicity and elicitor activity based on a ligand/receptor model. Two independent plant receptors exist in the first hypothesis, one of which recognizes a domain in the C-terminal half of NIP1. The consequence is H^+ -ATPase stimulation in both resistant and susceptible barley cultivars. The other one is the product of the *Rrs1* gene that probably recognizes another domain of the NIP1 protein, resulting in the induction of the plant defence response. In the second hypothesis, NIP1 interacts with a single type of receptor. In resistant plants, this receptor is encoded by the *Rrs1* gene and mediates both stimulation of the H^+ -ATPase and induction of defence reactions. In contrast, because of differences in the binding or effector site, the *rrs1*-encoded receptor of susceptible plants only stimulates H^+ -ATPase activity upon NIP1 binding. In the third hypothesis, the NIP1 receptor is not encoded by *Rrs1* and is expressed in both resistant and susceptible plants, thus leading to H^+ -ATPase stimulation upon binding. Defence reactions are induced if the product of the *Rrs1* gene, a downstream component of the signal

transduction pathway, interacts with the receptor, whereas in *rrs1* plants specific interaction or signal transduction does not occur (Knogge, 1996).

In situ hybridization using cross-sections of leaves inoculated with an avirulent fungal race or treated with NIP1 revealed that the accumulation of PR5 mRNA occurs in the mesophyll but not in the epidermis of *Rrs1* plants (Schmelzer and Knogge, unpublished data). In addition, preliminary data using NIP1-treated, separated leaf tissues indicate that the presence of both epidermis and mesophyll is required for PR5 mRNA induction (Knogge, unpublished data). This suggests that the putative NIP1 elicitor receptor is localized in the epidermis where a primary defence reaction may occur, which includes the generation of a signal that triggers PR protein synthesis in the mesophyll. Future research is aimed at the identification of the NIP1 receptor(s) and at the dissection of the signal pathways leading to the induction of either resistance or susceptibility using the native NIP1 and the synthetic peptides.

Avirulence Genes from *Magnaporthe grisea*

The filamentous heterothallic ascomycete, *Magnaporthe grisea*, causes blast disease on more than 50 different graminaceous species including rice (*Oryza sativa*), finger millet (*Eleusine coracana*) and weeping lovegrass (*Eragrostis curvula*). *M. grisea* has a very broad host range, but typically individual isolates can infect only one or a few grass species. Among pathogens of rice, hundreds of races were distinguished based on their ability to infect particular rice cultivars.

In a successful infection, the fungus produces an appressorium and a penetration peg that pierces through the cuticle and the epidermal cell wall. The fungus subsequently grows intracellularly in epidermal cells and colonizes adjacent mesophyll cells. Conidiophores differentiate five to seven days later, and thousands of new conidia are released from the lesions to reinstate the disease cycle (Valent and Chumley, 1991). In contrast, in the interaction of a weeping lovegrass isolate with a non-host, rice, the fungus failed to enter plant cells from half of the appressoria produced. Even at successful infection sites, no fungal spreading occurred from the first invaded epidermal cells which rapidly exhibit granular cytoplasm, browning and autofluorescence (Heath *et al.*, 1990a).

Since the discovery of the perfect stage of the fungus, a large number of avirulence genes (more than 15) have been identified by classical genetic analysis (Yaegashi and Asaga, 1981; Valent *et al.*, 1991; Valent and Chumley, 1991; Silué *et al.*, 1992a,b). It is not our intention to list all of the avirulence genes reported by different groups, but rather to focus on those that have been cloned and characterized in more detail.

PWL2, a host species-specific avirulence gene

PWL2, a gene that controls pathogenicity towards weeping lovegrass, was shown to segregate as a single gene among progeny obtained from a cross between a strain infecting weeping lovegrass and rice and a strain pathogenic only on rice. The occasional appearance of a few pathogenic lesions on weeping lovegrass inoculated with non-pathogenic progeny suggested that spontaneous mutation had occurred at the *PWL2* locus and that *PWL2* functions to prevent infection of weeping lovegrass (Sweigard *et al.*, 1995).

The *PWL2* gene was isolated by map-based cloning. Transformation of a weeping lovegrass pathogen containing a non-functional *PWL2* allele (see below) with *PWL2* confers non-pathogenicity to the strain, showing that *PWL2* is a dominant gene. In addition, *PWL2* is highly specific since it governs pathogenicity only towards weeping lovegrass but not towards rice or barley. *PWL2* is therefore referred to as a host species-specific avirulence gene since it prevents specifically the infection of weeping lovegrass, as avirulence genes *sensu strictu* control cultivar specificity within a particular host species (Sweigard *et al.*, 1995).

The intron-less *PWL2* gene encodes a protein of 145 amino acids including a putative signal sequence of 21 amino acids. *PWL2* contains a large percentage of glycine (18%) and charged amino acids (27.5%; Sweigard *et al.*, 1995). No homology was found between *PWL2* and other proteins from the database. Preliminary data indicate that the *PWL2* protein expressed in *Escherichia coli* does not seem to induce a hypersensitive response when infiltrated into weeping lovegrass tissue (Sweigard *et al.*, 1995). Upstream of the coding sequence, the nucleotide sequence of *PWL2* contains three 47 bp imperfect direct repeats whose function is not known. It appears that at least two copies of these repeats are necessary for *PWL2* to be functional. At the 3' end, *PWL2* also contains two copies of a 19 bp overlapping perfect direct repeat. Northern blotting indicated that *PWL2* is not expressed in strains grown on complete or minimal medium or under nitrogen-limiting conditions (Sweigard *et al.*, 1995).

Southern blotting and pathogenicity tests showed that rice field isolates which do not infect weeping lovegrass (24 out of 27 tested) contain one or more copies of *PWL2*. It is not known whether all of the *PWL2* homologues are functional. Weeping lovegrass pathogens have gained the ability to infect this plant species either by deleting 30 kb of the genomic region including *PWL2* (Valent and Chumley, 1994) or by mutating this gene (Sweigard *et al.*, 1995). A single base-pair exchange in the protein coding sequence, converting the aspartate-90 to an asparagine was found to abolish *PWL2* function. The amino acid sequence DKS of *PWL2* becomes NKS in the mutated protein, creating a glycosylation site. It is not known whether the loss of function of the mutated protein is due to glycosylation (Sweigard *et al.*, 1995).

The PWL multigene family

All strains isolated from different grass species such as *Digitaria*, pearl millet, finger millet or wheat contain at least one sequence ranging from strongly to weakly homologous to *PWL2*. However, there is no direct correlation between the ability of these pathogens to infect weeping lovegrass and the presence of *PWL2* homologues, suggesting that not all of the copies are functional (Kang *et al.*, 1995). A finger millet pathogen contains two *PWL2* homologues, *PWL1* and *PWL3*. Only *PWL1*, which does not map to the same location as *PWL2*, confers non-pathogenicity when transferred to a weeping lovegrass pathogen. A weeping lovegrass pathogen also contains a non-functional gene, termed *PWL4*, which is allelic to *PWL3* (Kang *et al.*, 1995).

Sequence analysis revealed that *PWL1*, *PWL3* and *PWL4* encode proteins of 147, 137 and 138 amino acids, respectively. The three proteins, like *PWL2*, contain a putative signal sequence of 21 amino acids and a high number of glycines (17 to 19% of all residues). Comparison of *PWL* amino acid sequences revealed an overall sequence identity of *PWL1*, *PWL3* and *PWL4* to *PWL2* of 75, 51 and 57%, respectively, with the highest degree of identity being localized in the C-terminal third of the proteins (Kang *et al.*, 1995). *PWL4*, but not *PWL3*, acts as a host specificity determinant when expressed from the *PWL1* or the *PWL2* promoter. This result suggests that *PWL4*, although functional, cannot be expressed from its own promoter, whereas *PWL3* does not encode an active protein.

The presence of *PWL1* in a strain appears to correlate with the induction of browning of weeping lovegrass cells beyond which the fungus does not grow (Heath *et al.*, 1990b). *PWL1* may therefore be involved in the elicitation of this response. In compatible interactions, *PWL* gene products are predicted to have a beneficial function since they have not only been maintained but also amplified in *M. grisea* strains showing different host specificities (Kang *et al.*, 1995). It is not known whether the different *PWL* genes match single plant resistance genes. One of the future research goals is to identify and isolate the putative resistance gene corresponding to *PWL2*. Since almost all rice pathogenic strains of *M. grisea* carry a *PWL2* gene, transformation of rice with this resistance gene might confer an efficient protection to this species.

The cultivar-specific avirulence gene AVR2-YAMO

The avirulence gene AVR2-YAMO, preventing *M. grisea* from infecting rice cultivar Yashiro-mochi, segregated as a single gene in progeny obtained from a cross between two fungal strains infecting different rice cultivars (Valent and Chumley, 1994). The gene was isolated by map-based cloning and found to encode a 223 amino acid protein which shares, in a short region, homology with the active centre of a neutral Zn²⁺-protease (see de Wit, 1995). In virulent

strains, a few point mutations were found within this putative active site, but direct evidence of protease activity of AVR2-YAMO has not been reported. Gain of virulence towards rice cultivar Yashiro-mochi can also result from an insertion of a 1.5-kb element into the AVR2-YAMO gene or deletions ranging from approximately 100 bp to over 12.5 kb (Valent and Chumley, 1994). Transformation of a strain virulent on Yashiro-mochi with AVR2-YAMO yields avirulence, thus confirming that this gene is a genuine avirulence gene (Valent and Chumley, 1994).

Resistance of rice cultivar Yashiro-mochi to AVR2-YAMO-carrying fungal strains is governed by a single blast resistance gene *Pi-62* (B. Valent, Delaware, 1994, personal communication). A weeping lovegrass pathogen carries an avirulence gene, *Avr1-YAMO*, which also prevents the fungus from infecting this rice cultivar (Valent *et al.*, 1991). *Avr1-YAMO* and AVR2-YAMO are not linked to each other. Genetic crosses of Yashiro-mochi with other rice cultivars will determine whether *Avr1-YAMO* and AVR2-YAMO interact with the same or different blast resistance genes.

Use of Avirulence Genes to Engineer Disease Resistance in Plants

A strategy was proposed, referred to as the 'two-component sensor system' (de Wit, 1992), to engineer disease-resistant plants utilizing fungal avirulence genes. By expressing an avirulence gene in plants containing the corresponding resistance gene, a hypersensitive response is expected to be induced thus preventing further microbial infection. Several conditions must be met for this strategy to be used successfully. One of them is the ability of transgenic plants to produce elicitor-active products of fungal avirulence genes. Only tobacco and tomato plants have so far been transformed with *Avr9* expressed from the 35S promoter (Hammond-Kosack *et al.*, 1994a; Honée *et al.*, 1995). Intercellular fluids from such transgenic plants contained an active protein inducing necrosis on Cf-9 tomato plants, thus showing that AVR9 is not only produced but also secreted and processed *in planta*. When the transgenic AVR9-producing Cf-0 plants were crossed with Cf-9 plants, fruits and seeds could be obtained (Hammond-Kosack *et al.*, 1994a; Honée *et al.*, 1995) and young F₁ seedlings developed normally. However, necrotic lesions appeared on cotyledons, on the primary leaves and eventually spread throughout the whole plantlet. Clearly, such plant death is not satisfactory. Expression either of the avirulence gene or the resistance gene or of both genes could be driven by an inducible promoter responding rapidly and locally to pathogen attack to circumvent this problem. Alternatively, mutated tomato plants could be used in which coexpression of resistance and avirulence genes will not lead to the induction of an HR but of other plant defence reactions. In this way, *nip1* could be an attractive candidate for this type of experiment since its gene product does not induce an HR.

Concluding Remarks

Unlike the products of bacterial avirulence genes, the elicitors from *C. fulvum* and *R. secalis* are secreted proteins directly inducing defence reactions in plants carrying the matching resistance genes. PWL2 is probably also a secreted protein but its mode of action in incompatible interactions remains to be determined. In contrast, AVR2-YAMO may be a protease releasing the elicitor from a yet unknown protein.

Fungal pathogens have followed different strategies to avoid the plant defence response and to colonize the plant successfully: complete deletion of avirulence genes (*Avr9*), or development of non-functional avirulence alleles (*Avr4*) or a combination of both (*PWL2*, *AVR2-YAMO*, *nip1*). However, avirulence genes have frequently been conserved suggesting they might also have an important beneficial role during compatible interactions. Except for *NIP1*, the intrinsic function of avirulence gene products during the infection process is unknown.

Many intriguing questions remain to be answered to prove the elicitor/receptor model for function of avirulence and resistance genes. Is AVR9 interacting directly with the Cf-9 protein? What are the structures of other receptors for avirulence gene products? Are second messengers involved in the signal transduction pathway leading to plant defence activation or are the elicitor/receptor complexes translocated to the nucleus? In the *C. fulvum*/tomato interaction, a number of plant defence reactions has been identified. Which responses are related to each other? How similar are they in other plant/pathogen interactions? One strategy to identify essential components involved in the activation of the resistance response is to mutate resistant plants. Mutations in genes required for the function of particular resistance genes have already been isolated in tomato and barley (see Schultze-Lefert *et al.*, Chapter 3 this volume; Hammond-Kosack *et al.*, 1994b; Freialdenhoven *et al.*, 1994, 1996). It is predicted that similar strategies will be followed in other species in order to demystify what occurs during the onset of plant resistance.

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The Molecular Genetics of Plant–Virus Interactions

18

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Resistance is the most effective and economical method of choice for the control of plant viruses and greater knowledge of the molecular interactions between host and virus will lead to novel approaches to gene manipulation and should result in better control of virus diseases in the future. An understanding of the molecular variation among virus strains is important in the development of host genotypes with durable resistance and provides insight into interactions with host plant genes. The development of a gene-for-gene model to describe interactions between plants and viruses requires a synthesis of information about variation in the virus and resistance genes in the host. Authenticated gene-for-gene relationships exist in a rather limited number of host–virus combinations. These include tomato/tomato mosaic virus (ToMV), tobacco/tobacco mosaic virus (TMV) and potato/potato virus X (PVX). In each case the molecular genetics of the relationship between virus and host have been elucidated in some detail.

Terminology

The concept of virulence/avirulence in plant–virus interactions is a terminology which requires some clarification. In plant virology the term virulence has often been used to describe the degree of symptom severity; for example, ‘virulent’ strains of TMV compared with ‘mild’ ones (Watanabe *et al.*, 1987). However, virulence has also been defined, perhaps more appropriately, as the ability to overcome resistance (Fraser, 1990). In this context, the virulence/avirulence concept has been extrapolated from the gene-for-gene hypothesis

for application in plant–virus interactions. There are examples of mutations in specific viral genes that break the resistance conferred by specific host genes, such as mutations in the 30-kDa protein gene of ToMV that overcome *Tm2* resistance in tomato (Meshi *et al.*, 1989). Used in this sense, an isolate of TMV that causes a hypersensitive local lesion (incompatible reaction) on tobacco would be avirulent, while an isolate causing systemic mosaic (compatible reaction) would be virulent regardless of the severity of the reaction. This is the context in which virulence/avirulence is used throughout this chapter.

Tomato Mosaic Virus

Tomato mosaic (ToMV) is the most common viral disease of tomato, caused by a mechanically transmitted positive-sense RNA virus that is closely related to tobacco mosaic virus (TMV). A postulated gene-for-gene relationship was first presented by Pelham (1969). Rast (1975) subsequently added another pathotype resulting in a model with five virus pathotypes differentiated by their reactions on four tomato genotypes with various combinations of *Tm* genes (Table 18.1).

Although susceptible to some strains, cultivars which carry the *Tm1* gene have been useful because of the prevalence in nature of incompatible strain 0. The *Tm2*² gene is widely used today and still remains effective against ToMV in commercial plantings worldwide. It has been possible to infer the general mechanism for viral resistance for some genes by determining whether resistance inhibits viral replication, as in *Tm1* (Fraser and Loughlin, 1980), or cell-to-cell movement, as in *Tm2* (Motoyishi and Oshima, 1977). The molecular basis of these and other resistance genes is being investigated further using recombinant DNA techniques.

Using near-isogenic lines of tomato, restriction fragment length polymorphism (RFLP) markers have been found near both the *Tm1* and *Tm2* genes in tomato (Young *et al.*, 1988). These have been used as a starting point for map-based cloning of these genes. Meshi *et al.* (1989) examined the nucleotide

Table 18.1. Relationships between genes for resistance in tomato with strains of tomato mosaic virus.

Tomato genotype	Tomato mosaic virus strains				
	0	1	2	1.2	2 ²
(+/+)	S	S	S	S	S
<i>Tm1/Tm1</i>	T	S	T	S	R
<i>Tm2/Tm2</i>	R	R	S	S	R
<i>Tm2</i> ² / <i>Tm2</i> ²	R	R	R	R	S

S = susceptible; T = tolerant; R = resistant.

sequences of two ToMV mutants capable of overcoming *Tm2* resistance in tomato and sequence analysis revealed two different substitutions of two amino acids in the 30-kDa movement proteins of the mutants compared with the wild type. Both sets of substitutions were required to overcome *Tm2* resistance which suggested that the *Tm2* resistance response is elicited by the 30-kDa movement protein.

As a means of studying the mechanism of *Tm2*² resistance, cDNA clones that were able to break *Tm2*² resistance were selected from a library of the ToMV strain 2² genome (Weber *et al.*, 1993). Chimeric full-length viral cDNA clones that combined parts of the wild-type ToMV and ToMV2² were constructed under the control of the cauliflower mosaic virus 35s promoter. Using these clones in cDNA infection experiments, it was demonstrated that alteration in the 30-kDa movement protein of ToMV2² resulted in the ability to overcome the *Tm2*² resistance gene. DNA sequence analysis revealed four amino acid exchanges between the 30-kDa proteins from the wild-type ToMV and ToMV2². Different combinations of these amino acid exchanges were introduced in the genome of wild-type ToMV to clarify the involvement of the altered amino acid residues in the resistance-breaking properties of the ToMV2² movement protein. Only one mutant strain which contained two amino acid substitutions was able to multiply in *Tm2*² tomato plants. Both amino acid exchanges were found in the carboxy-terminal region of the movement protein.

These observations suggest that the resistance conferred by the *Tm2*² gene against ToMV depends on specific recognition events in this plant–virus interaction rather than interfering with fundamental functions of the 30-kDa protein.

Tobacco Mosaic Virus

The *N'* gene, a single dominant gene originating from *Nicotiana sylvestris*, is associated with a hypersensitive reaction (HR) directed against most strains of tobamoviruses. Saito *et al.* (1989) first demonstrated the involvement of the coat protein sequence in the induction of the *N'* gene-mediated HR by substituting the coat protein gene of a strain of TMV, which did not induce *N'* gene HR, into the genome of an HR-inducing strain. The hybrid virus was incapable of inducing HR in *N'* gene hosts. They concluded that the induction of the *N'* gene HR was localized in the open reading frame (ORF) of the coat protein. Furthermore, Knorr and Dawson (1988) demonstrated that a single nucleotide substitution in the coat protein ORF of a mutant strain could prevent HR induction in an *N'* gene host. The molecular mechanisms responsible for HR induction in *N. sylvestris* were examined further by Culver *et al.* (1991). Mutants of TMV were used to demonstrate that it was the coat protein and not the RNA that was responsible for induction of the *N'* gene HR.

The dominant *N* gene was introduced into TMV sensitive (TMV^S) *Nicotiana tabacum* from the related TMV resistant (TMV^R) species *N. glutinosa*. TMV infection of NN tobacco induces HR within 48 h of infection and TMV is restricted to the region immediately surrounding the induced necrotic lesions. In contrast, TMV^S tobacco cultivars lacking the *N* gene (*nn* tobacco) allow TMV to spread systemically and develop mosaic symptoms. TMV encodes four proteins: two are required for viral replication (126 kDa and 183 kDa), one for cell-to-cell movement (30 kDa), and one is required for viral RNA encapsidation (17.5 kDa). The coat protein does not appear to be involved in induction of HR in plants with the *N* gene. TMV mutants with a deletion of the entire coat protein ORF induce normal necrotic lesions which were indistinguishable from those induced by the wild-type virus (Dawson *et al.*, 1988; Saito *et al.*, 1989). In addition, a mutant with an alteration in the coat protein translational start codon, preventing the production of coat protein but maintaining an intact coat protein ORF, did not effect the induction of necrotic lesions in *N* gene plants (Culver and Dawson, 1989). Thus the coat protein and its ORF are not directly involved in the induction of the *N* gene HR.

Although the TMV avirulence gene corresponding to *N* has not been identified conclusively, one study suggests that the 126-kDa replicase protein is required for HR induction in NN tobacco. TMV-induced HR is accompanied by the induction of defence mechanisms in NN plants but not in *nn* plants (Baker *et al.*, 1994). The relationships between TMV activation of HR, the induction of defence responses and the prevention of viral spread have not yet been established.

It has been hypothesized that the product of the *N* gene may be capable of recognizing TMV and initiating subsequent defence responses. As such, *N* may be one of the first critical genes required in the pathway for resistance to TMV. The *N* gene was recently isolated by tagging with the maize transposon *Ac* (Dinesh-Kumar *et al.*, 1995) and this should allow elucidation of the molecular and biochemical basis of *N*-mediated resistance to TMV. The isolation of the *N* gene was confirmed by a transformation experiment in which a TMV-compatible genotype of tobacco was transformed to incompatibility with a genomic DNA fragment carrying *N*. Sequence analysis of the *N* gene showed that it encoded a protein with an amino-terminal domain similar to that of the cytoplasmic domains of the *Drosophila* Toll protein and the interleukin-1 receptor in mammals, a putative nucleotide-binding site and 14 imperfect leucine-rich repeats. The presence of these functional domains in the predicted *N* gene product is consistent with the hypothesis that the *N* resistance gene functions in a signal transduction pathway. Similarities of *N* to Toll and the interleukin-1 receptor suggest a similar signalling mechanism leading to rapid gene induction and TMV resistance (Dinesh-Kumar *et al.*, 1995).

Plants with the *nn*, *n'n'* genotype are susceptible to most strains of TMV resulting in systemic mosaic symptoms. A series of deletions made to examine the secondary functions of the coat protein resulted in mutants which had

distinct symptomatology, including those that induced local necrosis in Xanthi tobacco, which has the *nn*, *n'n'* genotype and does not normally respond with localized necrosis to wild-type TMV (Dawson *et al.*, 1988). Only specific deletions in the coat protein induce necrosis in the *nn*, *n'n'* genotype plants, and the induction of necrosis in hosts not normally susceptible to TMV suggests that this form of necrotic response is non-specific and does not involve interaction with a host resistance gene.

Potato Virus X

The molecular basis of the interaction between isolates of potato virus X (PVX) and resistance genes *Nb*, *Nx* and *Rx* in potato has been well studied (Table 18.2). All known isolates of potato virus X (PVX), with the exception of a South American isolate PVX_{HB}, induce an extreme resistance response in potato carrying the *Rx* gene and elicit the production of necrotic lesions on *Gomphrena globosa*. PVX_{HB} establishes systemic infection on *Rx* genotypes of potato and infects inoculated leaves of *G. globosa* without lesion formation. *Rx*-mediated resistance is elicited by and requires the presence of a threonine residue at position 121 as determined for isolate PVX_{CP4}. The resistance is an induced response expressed in protoplasts of potato with the *Rx* genotype (Goulden *et al.*, 1993). There is now evidence, based on the analysis of PVX_{CP4}/PVX_{HB} hybrids, that the elicitation of lesions on *G. globosa* also requires the presence of a threonine residue at position 121 of the viral coat protein (Goulden and Baulcombe, 1993). The lesion-forming phenotype was not associated with the ability to accumulate in the infected plant and it was therefore proposed that a homologous component of both potato carrying *Rx* and *G. globosa* interacts with a feature of the PVX coat protein. A defence response is induced in the plant cell as a consequence of this molecular interaction.

The *Rx* resistance gene is expressed in whole plants and in protoplasts. *Rx*-mediated resistance in protoplasts reduces the accumulation of all PVX

Table 18.2. The response of strains of PVX on potato varieties with different resistance genotypes.

Potato genotype	PVX strain				Strain HB
	Group 1 (DX)	Group 2 (CP2)	Group 3 (UK3)	Group 4 (CP4)	
<i>Nx, Nb, rx</i>	HR	HR	HR	S	S
<i>nx, Nb, rx</i>	HR	HR	S	S	S
<i>Nx, nb, rx</i>	HR	S	HR	S	S
<i>nx, nb, Rx</i>	ER	ER	ER	ER	S

HR = hypersensitive resistance; S = susceptible; ER = extreme resistance.

RNA species after a lag of 8 h after inoculation. The virulence of isolate PVX_{HB} to *Rx* is associated with an alteration of the coat protein gene. In the case of isolates PVX_{UK3} and PVX_{CP4} a frame-shift mutation in the coat protein gene had no effect on the interaction with *Rx* cultivars but compromised the *Rx*-mediated resistance to isolate PVX_{CP4} (Köhm *et al.*, 1993). *Rx*-mediated resistance was induced when the PVX coat protein was produced in infected cells and the induced resistance mechanism was also effective against the unrelated cucumber mosaic virus (CMV).

The coat protein determines whether isolates of PVX are virulent or avirulent on *Rx* cultivars of potato. Isolates with the coat protein of PVX_{HB} are virulent; those with the coat protein of PVX_{UK3} elicit an extreme resistance in the *Rx* potato that prevents virus accumulation even on the inoculated leaf (avirulent). Goulden *et al.* (1993) describe the analysis of a series of hybrid and mutant isolates of PVX_{HB} and PVX_{CP4} which were inoculated to plants and protoplasts of *Rx* and *rx* cultivars of potato. They concluded from the virulence phenotypes of these isolates that elicitation of the resistance was affected by amino acids 121 and 127 of the viral coat protein, with codon 121 being the major determinant. PVX_{HB} and hybrid or mutant isolates with lysine and arginine at positions 121 and 127 were able to overcome the resistance of *Rx*, whereas those with threonine and arginine were avirulent both on plants and in protoplasts. Viral isolates with single mutations at either codon 121 or 127 were less infectious than the wild type or double-mutant isolates; although in protoplasts of the susceptible cultivar of potato, they accumulated as well as the wild-type virus. Taken together, these data suggest that amino acids 121 and 127 affect a feature of the viral coat protein which may interact with cellular components involved in the spread of PVX and with the product of the *Rx* resistance gene (Goulden *et al.*, 1993).

The coat protein gene of potato virus X is also known to affect the outcome of interactions between different strains of the virus and potato plants carrying the *Nx* resistance gene. In order to analyse the role of the coat protein in interactions with *Nx* hosts, Santa Cruz and Baulcombe (1993) used the potato virus X strain PVX_{DX}, which induces an HR on potato cultivars carrying the *Nx* resistance gene, and the strain PVX_{DX4}, which was originally derived from PVX_{DX} and which overcomes *Nx*-mediated resistance. Sequencing of cloned coat protein genes representing the strains PVX_{DX} and PVX_{DX4} showed that they differed at a single nucleotide. This change resulted in the substitution of glutamine at position 78 in the PVX_{DX} coat protein for proline in PVX_{DX4}. They constructed hybrid viral genomes by replacing the coat protein gene of a full-length clone of isolate PVX_{UK3} with the corresponding sequence from either PVX_{DX} or PVX_{DX4}. Progeny virus, derived from *in vitro* transcripts of these hybrid clones, showed that the single nucleotide difference between the coat protein genes of isolates PVX_{DX} and PVX_{DX4} was sufficient to alter the outcome of the interaction between the virus and potato plants carrying the resistance gene *Nx*. Additional coat protein mutants generated *in planta* from

transcript-derived inocula induced an intermediate host response on Nx potato cultivars, which is influenced by the presence of a second, PVX-specific, resistance gene in the host plant genome.

Chapman *et al.* (1992) described the effects of various mutations introduced into the coat protein gene of a full-length PVX cDNA clone from which infectious RNA transcripts have been produced (Kavanagh *et al.*, 1992). The results showed that changes in the coat protein gene can affect, either directly or indirectly, virion morphology, plant symptoms, viral pathogenicity, and accumulation in protoplasts of positive- but not negative-strand RNA. The role of the coat protein of PVX was investigated by site-directed mutagenesis of the coat protein gene. Mutant viruses with in-frame deletions of the 5' end of the coat protein gene were capable of systemically infecting plants, but produced virions with atypical morphology. Viruses with a frame-shift mutation near the 5' end or with deletions in the central part of the coat protein gene failed to accumulate at detectable levels, even in the inoculated leaf. In protoplasts, mutants that infected systemically either had a wild-type phenotype or showed a small reduction in accumulation of genomic RNA. The other mutants, which did not accumulate in the inoculated leaf, were unaffected in genomic RNA accumulation for 8 h after inoculation, but after 16 h and later, they accumulated less genomic RNA than wild-type virus. None of the mutations had an effect on accumulation of negative-strand RNA. The data indicated that efficient accumulation and spread of PVX, even in the inoculated leaf, require coat protein production and encapsidation of the viral RNA.

Application of Molecular Genetics for Crop Improvement

The ability to make defined mutations in viral genomes and then to examine the effects of the mutations on the functioning of the virus has allowed precise mapping of viral sequences involved in replication, regulation and gene expression. These technologies are now significantly advancing the understanding of virus–host interactions. Molecular analyses of plant–virus interactions have also resulted in the discovery and exploitation of pathogen-derived resistance, where resistance results in plants expressing components of the viral genome.

The use of pathogen-derived resistance to produce virus resistant plants was first demonstrated with TMV, initially using the coat protein gene (Powell-Abel *et al.*, 1986) and later using the gene encoding the viral replicase (Golemboski *et al.*, 1990). Variable levels of resistance have been observed in plants transgenic for TMV coat protein and plants were resistant only to low levels of TMV inoculum and were not resistant when the inoculum was applied as TMV RNA (Nelson *et al.*, 1987), in contrast to plants transgenic for the coat protein gene of PVX which are equally resistant to infection by virions or RNA (Hemenway *et al.*, 1988).

In general, pathogen-derived resistance tends to be specific only for the virus from which the transgene was isolated. For example, plants transformed with the coat protein of the U1 strain of TMV were most resistant to that strain of TMV and its close relatives, and exhibited less resistance to other tobamoviruses (Nejdat and Beachy, 1990). There is also evidence that the resistance of plants transformed with viral replicase is even more specific than that of plants transformed with coat protein genes. For example, plants transgenic for the 54-kDa replicase gene of the U1 strain of TMV, whilst highly resistant to that strain and mutants derived from it, were not resistant to other tobamoviruses (Golemboski *et al.*, 1990). An exception, however, was found in plants transgenic for the 183-kDa replicase gene of TMV which is interrupted by a bacterial insertion element. A number of lines transgenic for this gene were resistant not only to the strain from which the gene was isolated but also to distantly related tobamoviruses including tomato mosaic tobamovirus, tobacco mild green mosaic tobamovirus, green tomato atypical mosaic tobamovirus and ribgrass mosaic tobamovirus (Donson *et al.*, 1993).

The broadest resistance in transgenic plants has been found in those transformed with viral movement proteins. Plants transgenic for a deleted version of the TMV movement protein were resistant to a wide range of tobamoviruses, including tobacco mild green mosaic virus and sunn hemp mosaic virus (Lapidot *et al.*, 1993; Cooper *et al.*, 1995).

Several reports have described virus resistance resulting from transgenic expression of the putative RNA polymerase of plant RNA viruses. In order to test whether dominant negative mutations can be used to obtain resistance in transgenic plants and analyse functions of replicase in PVX, Longstaff *et al.* (1993) introduced three types of mutation into the sequence encoding the GDD motif of the putative replicase component of PVX. All three mutations rendered the viral genome completely non-infectious when inoculated into *Nicotiana clevelandii* or into protoplasts of *N. tabacum* (cv. Samsun NN). In order to test whether these negative mutations could inactivate the viral genome *in trans*, the mutant genes were expressed in transformed *N. tabacum* (cv. Samsun NN) under control of the 35s RNA promoter of cauliflower mosaic virus and the transformed lines were inoculated with PVX. There was no effect on susceptibility to PVX in 10 lines tested in which the GDD motif was expressed as GAD or GED. In two of four lines transformed to express the ADD form of the conserved motif, the F₁ and F₂ progeny plants were highly resistant to infection by PVX, although only to strains closely related to the source of the transgene. The resistance was associated with suppression of PVX accumulation in the inoculated and systemic leaves and in protoplasts of the transformed plants, though some low-level viral RNA production was observed in the inoculated but not the systemic leaves when the inoculum levels were high. These results suggest that for a plant virus, resistance may be engineered by expression of dominant negative mutant forms of viral genes in transformed cells.

Concluding Remarks

The examples described support the basic assumption of the gene-for-gene hypothesis that single dominant genes are involved in virus perception and subsequent induction of plant defence responses. The most likely role of genes governing such critical control points in the resistance pathway is that of a receptor for a ligand produced by the virus. In the case of resistance to TMV, the product of the *N* gene contains sequence motifs that suggest it could be a receptor molecule or another important component of a signal transduction pathway.

Because of the small size of the viral genome relative to bacteria and fungi, there may be greater potential for designing durable resistance to viruses than there is to other plant pathogens. For example, a combination of resistance genes that recognize different features of the coat protein with others that recognize the replicase gene could present an insurmountable challenge for adaptation of a viral pathogen. Cumulative data on the effectiveness of different pathogen-derived resistance strategies across several virus groups in several plant species suggest that this approach will not provide the final answer for virus protection. Although there are reports of near immunity, in general the level of resistance is less than desirable. Nevertheless, it is clear that a detailed understanding of the molecular genetics of the host–virus interaction is essential for further progress to be made.

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Molecular Genetics of Disease Resistance: an End to the 'Gene-for-Gene' Concept?

19

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The 'gene-for-gene' hypothesis as proposed by Flor has been the fundamental concept that has shaped theory concerning the interactions between plants and pathogens (Flor, 1971). This chapter will aim to illustrate how this concept is being reformulated in the light of recent developments in understanding the molecular structure of plant disease resistance genes, including studies that utilize both natural variation and artificially induced mutation. Initial clues suggest that disease resistance is commonly the result of a biochemical pathway from perception to defence response, and that the pathogen specificity of a pathway is governed by only a few components. Future discoveries will no doubt reveal how disease resistance has evolved in plants by an integration of biochemical processes into a general network of damage-induced responses.

The Gene-for-Gene Hypothesis

Flor's ground breaking work (Flor, 1946, 1947, 1955) defined certain genetic parameters concerning the interaction between a microbial pathogen and its host plant. In the modern interpretation, genes exist in the plant (so-called R-genes in more recent literature) that enable the initiation of a disease resistance response when challenged with an appropriate pathogen isolate. The absence of these host genes would allow the pathogen to invade because the host would be incapable of triggering a defence response. However, if a plant contained a particular resistance gene, it only detected certain pathogen isolates (or races) whereas other isolates failed to elicit a resistance response. This suggested that pathogen isolates carry genes, the products of which interact

with corresponding host gene products to elicit the disease resistance response. These pathogen genes have conventionally been called *avirulence* (or *avr*) genes. The absence of the *avr* gene from a pathogen isolate will allow host invasion even if a resistance gene is present. This interaction is summarized in Table 19.1. A resistance response will only occur when the pathogen produces a gene product that creates a feature that can be detected by the presence of a specific resistance gene in the plant.

A simple model of a resistance gene would be a molecule that can detect the *avr* gene product (or metabolite that it is responsible for producing) directly and transmit this pathogen recognition signal to a defence response mechanism. Figure 19.1 illustrates several possible scenarios. The avirulence signal could potentially be any extracellular molecule or surface feature produced by the pathogen. It would be essential for the resistance gene to have a domain that enables detection of the *avr* signal, most likely extracellular and anchored via a membrane-spanning domain. This would in turn be connected to a protein domain involved in signal transduction. Protein kinases are often involved in such signal transduction pathways. These structures would conform perfectly with the gene-for-gene concept because if either component were lacking, then no disease response mechanisms would be initiated. Important aspects of this model which need to be confirmed are: whether the same host protein is responsible for detection and for triggering signal transduction leading to a host defence response; whether the AVR detection domain of a resistance gene can be cytoplasmic instead of extracellular (as shown in Fig. 19.1); and whether a single resistance gene can be involved in the detection of more than one related, or unrelated, *avr* gene product. Equally, a particular AVR signal could be detected by more than one resistance gene in either the same or different host plants. None of these possible modifications is necessarily a contradiction of the gene-for-gene hypothesis, in that they all propose the presence of a plant gene involved in the specific detection of an AVR signal from the pathogen.

Having detected invasion by a pathogen, the plant must respond in such a way as to prevent the spread of that pathogen, in order to reduce cellular damage resulting in loss of active tissues. This would include loss of photosynthetic capacity and use of photosynthate by a pathogen. In these types of

Table 19.1. The basic premise of the gene-for-gene hypothesis.

Host plant	Pathogen	
	Avirulence gene present	Avirulence gene absent
Resistance gene present	Interaction leading to resistance	No interaction, pathogen growth allowed
Resistance gene absent	No interaction, pathogen growth allowed	No interaction, pathogen growth allowed

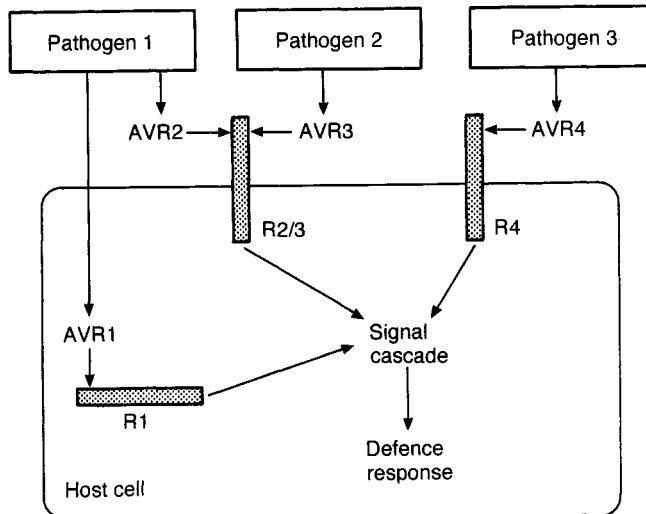


Fig. 19.1. Potential routes in host cells for the detection of avirulence (AVR) signals produced by the pathogen *avr* gene and signalling of defence responses triggered by the gene product of a host *R*-gene. AVR2, 3 and 4 are extracellular signals; AVR1 is intracellular. R1 is cytoplasmic and capable of detecting AVR1. R2/3 is membrane spanning and capable of detecting more than one AVR signal extracellularly. R4 is membrane spanning and detects AVR4 extracellularly. Pathogens 2 and 3 produce one AVR signal, but pathogen 1 produces 2.

interaction, the most common host resistance response is localized cell death at the point of penetration by the pathogen, conventionally called the hypersensitive response (HR). The manifestation of this response could be the production of a clearly visible lesion on the plant surface or limited to the death of a single cell. A single plant can produce very different responses when challenged with different isolates of the same pathogen. For example, Holub *et al.* (1994) described the interaction between the crucifer, *Arabidopsis thaliana*, and the oomycete, *Peronospora parasitica* (downy mildew). When challenged with parasite isolate *Emoy2*, a spreading lesion, or necrotic pit, was produced by host accession Niederzenz; whereas most interactions resulted in localized cell death, or necrotic flecking, involving only penetrated host cells. Single genes have been identified that are involved in the various interaction phenotypes (Holub *et al.*, 1994; Holub and Beynon, 1997). Nevertheless, important physiological differences must exist between such phenotypes in the way that the host responds to detection of the different parasite isolates.

One of the earliest detectable responses in an incompatible interaction is the production of a burst of superoxide, possibly involving NADPH oxidase (Lamb, 1994). It is likely, therefore, that the detection of an AVR signal by a resistance gene results in the induction of a signal transduction pathway

resulting in an oxidative burst. As a consequence of this, plant cell walls are strengthened, cellular decompartmentalization occurs and phytoalexins (antimicrobial compounds) and pathogenesis-related proteins (e.g. chitinase and glucanase) are produced in surrounding cells. The mechanisms by which these responses are induced or regulated are not understood. The simple model of the gene-for-gene interaction described in Fig. 19.1 must, therefore, include a signal transduction pathway leading to the oxidative burst and gene induction in surrounding cells.

Several groups have reported the cloning of disease resistance genes in recent months and, although common themes can be elucidated, they have proven to have a wide range of structures. Instead of describing these genes in chronological order of discovery, the following section will present the genes in a way that will intertwine their functions and demonstrate that various systems could be used to result in a resistance response.

Disease Resistance Genes

Xa21: a complete model resistance gene?

Song *et al.* (1995) described the cloning of *Xa21*, a rice gene which confers resistance to *Xanthomonas oryzae* pv. *oryzae* (*Xoo*) race 6, the causal agent of bacterial blight. The gene is composed of several discrete domains, the functions of which can be proposed based on homology to previously studied genes (Fig. 19.2). An N-terminal domain is characteristic of a signal sequence, suggesting that the protein is targeted to an extracellular location. This is followed by 23 imperfect copies of a 24 amino-acid leucine rich repeat (LRR). LRR motifs have been implicated in protein/protein interactions (Kobe and Deisenhofer, 1994). After the LRR, the protein contains a structure likely to be a membrane-spanning helix, suggesting that although the N-terminal of the protein is extracellular the C-terminal is intracellular. Here it is possible that the AVR signal from *Xoo* interacts with the LRR domain and that the interaction occurs extracellularly. Finally, the C-terminal domain is indicative of a protein kinase and contains conserved sequences that would suggest that it has serine-threonine specificity. Therefore, it is likely that this kinase domain is responsible for passing the information that the LRR has detected an AVR signal on to a putative intracellular disease-resistance response pathway.

Cf-2 and Cf-9: where is the signal transduction component?

The structures of resistance genes *Cf-2* and *Cf-9* from tomato have recently been published (Jones *et al.*, 1994; Dixon *et al.*, 1996). These genes confer

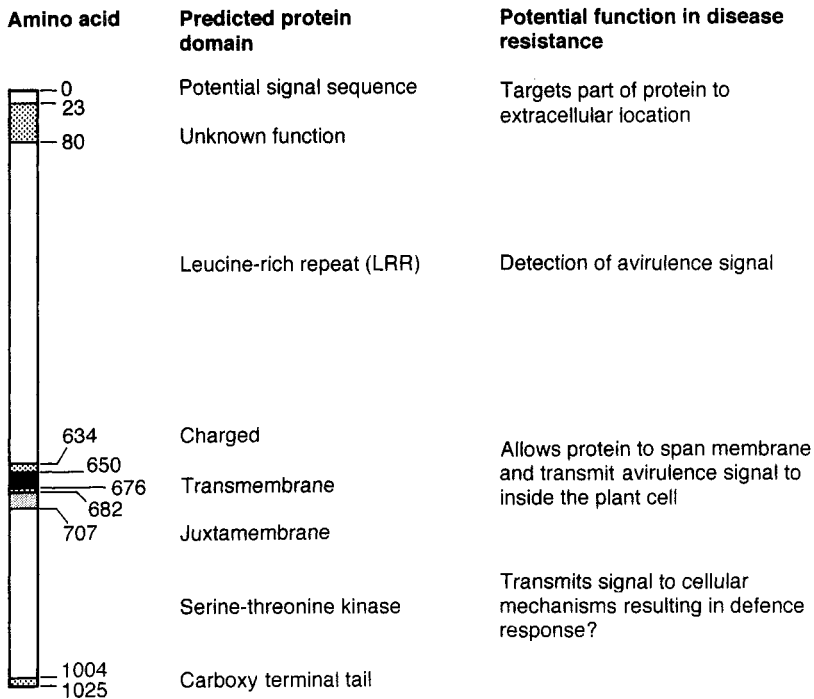


Fig. 19.2. Predicted protein structure of the rice disease resistance gene, *Xa21* (detail taken from Song *et al.*, 1995).

resistance to isolates of the fungal pathogen *Cladosporium fulvum* (grey mould) that carry the avirulence genes *Avr2* and *Avr9*, respectively. The *Cf-2* locus contained two functional genes (an issue that will be discussed below) that were highly similar to one another and to the *Cf-9* gene (Dixon *et al.*, 1996). The basic structure of these genes shares much with that of *Xa21* (Table 19.2). The N-terminal contains a putative signal peptide followed by an LRR domain with 33 perfect and five imperfect repeats (in the case of *Cf-2*). Therefore, like *Xa21*, the LRR domain is extracellular and has the potential to be glycosylated. The extracellular location of these LRR domains is completely consistent with the structure and location of the *Avr9* product. The active *Avr9* product is a small, cysteine-rich peptide (van Kan *et al.*, 1991), the purified form of which can elicit the disease resistance response in only those tomato plants containing the *Cf-9* gene. This would imply a direct interaction between *AVR9* and *Cf-9*, probably mediated via the LRR domain. The LRR domain is followed by a hydrophobic stretch of amino acids consistent with a membrane-spanning region. Again, this is similar to *Xa21* and suggests that the extracellular LRR domain is anchored to the plant cell membrane. Unlike *Xa21*, however, *Cf-2* and *Cf-9* do not contain an intracellular kinase domain and consequently

Table 19.2. A comparison of the structural features of disease resistance genes.

Host		Pathogen		Resistance gene product structure				Reference
Plant	Gene	Name	Type	N-terminal feature	Leucine rich repeat	Membrane association	Signal transduction	Primary author
Rice	<i>Xa21</i>	<i>X. oryzae</i>	Bacterial	Signal sequence	✓	Spanning	Kinase	Song, 1995
Tomato	<i>Cf-2, Cf-9</i>	<i>C. fulvum</i>	Fungal	Signal sequence	✓	Spanning	None	Jones, 1994; Dixon, 1996
Tomato	<i>Pto</i>	<i>P. syringae</i>	Bacterial	Myristillation?	×	Associated?	Kinase	Martin, 1993
<i>Arabidopsis</i>	<i>RPS2</i>	<i>P. syringae</i>	Bacterial	Leucine zipper	✓	Associated?	Neucleotide binding	Mindrinis and Bent 1994
<i>Arabidopsis</i>	<i>RPM1</i>	<i>P. syringae</i>	Bacterial	Leucine zipper	✓	Associated?	Neucleotide binding	Grant, 1995
Tobacco	<i>N</i>	TMV	Virus	Toll like	✓	Cytoplasmic	Neucleotide binding	Whitham, 1994
Flax	<i>L⁶</i>	<i>M. lini</i>	Fungal	Signal sequence? + toll?	✓	?	Neucleotide binding	Lawrence, 1995
Maize	<i>Hm1</i>	<i>C. carbonum</i>	Fungal	None	×	None	None	Johal, 1992

require another component to connect with a signal transduction pathway that could lead to a defence response.

Pto: how does it interact with the AVR signal?

The gene *Pto* confers resistance in tomato plants to strains of the bacterial pathogen *Pseudomonas syringae* pv. *tomato* (the causative agent of bacterial speck) that express the avirulence gene *avrPto*. Martin *et al.* (1993) cloned *Pto* and showed that the gene encodes a protein kinase which specifically phosphorylates serine and threonine residues. The gene contains no signal sequence, LRR structures or membrane-spanning domain. This suggests a cytoplasmic location for the gene product, but the N-terminal region does contain a potential myristoylation site that may imply that the protein is in fact membrane associated. *Pto* can obviously be part of a signalling pathway leading to a resistance response but lacks any obvious feature that would suggest a means for interacting directly with an *avr* gene product. Table 19.2 shows how the structure of this gene compares with those of *Xa-21* and *Cf-2/9*. Genes such as *Cf-2* lack a kinase domain but contain LRRs, whereas *Pto* is the converse. Genes like *Cf-2* may detect the AVR signal and pass that message to membrane-associated kinases like *Pto* that could then initiate a signal pathway leading to disease resistance.

RPS2, RPM1, N and L⁶: variations on a theme

RPS2 and *RPM1* are genes in *Arabidopsis* that confer resistance to the bacterial pathogens *Pseudomonas syringae* pv. *tomato* expressing the avirulence gene *avrRpt2* (Bent *et al.*, 1994; Mindrinis *et al.*, 1994) and *Pseudomonas syringae* pv. *maculicola* expressing the avirulence gene *avrRpm1* (Debener *et al.*, 1991; Grant *et al.*, 1995), respectively. The tobacco gene *N* confers resistance to the viral pathogen, tobacco mosaic virus (TMV) (Whitham *et al.*, 1994). And, *L⁶* is a flax gene that confers resistance to the fungal pathogen, *Melampsora lini* (the original system used by Flor to define the gene-for gene hypothesis) (Lawrence *et al.*, 1995). Table 19.2 includes all four genes in the comparison of the resistance genes.

RPS2, *RPM1*, *L⁶* and *N* all contain LRR domains, the specific structures of which may, however, be very different. *RPS2*, *RPM1* and *N* all appear to be located cytoplasmically and *L⁶* may be membrane associated. All four genes contain a nucleotide-binding domain which is commonly found in proteins known to bind ATP/GTP. Binding of such nucleotides could be important in signalling in the cell and, hence, being part of a signal transduction pathway leading to disease resistance. *N* and *L⁶* contain amino acid sequences toward their N-terminal that show homology to the cytoplasmic domains of

the *Drosophila* Toll protein and the human interleukin-1 receptor (IL-1R). IL-1R is involved in the translocation of a transcription factor that results in the synthesis of a range of defence and signalling proteins involved in immune, inflammatory and acute phase responses (Baeuerle, 1991). Such structures would be consistent with the role of N and L⁶ in signalling to the defence-related genes that the appropriate pathogen is present. RPS2 and RPM1 have structures at their N-terminal that are reminiscent of proteins containing leucine zippers; such sequences are involved in protein dimerization.

Hm1: a different class of resistance gene

Hm1 controls resistance in maize to the fungal pathogen *Cochliobolus carbonum* (Johal and Briggs, 1992). The presence of this gene makes plants resistant to race 1 isolates of the fungus that produce a pathogenicity factor called HC-toxin. Hence, *Hm1* differs markedly from the previously described resistance genes because it does not appear to involve a signal transduction pathway, and the lack of the ability in the pathogen to produce the toxin renders it unable to invade the host. *Hm1* codes for a reduced nicotinamide adenine dinucleotide phosphate (NADPH)-dependent HC-toxin reductase enabling plants that contain it to detoxify the HC-toxin and, hence, preventing invasion by the pathogen.

A summary of resistance gene structure

With the exception of *Hm1*, the other resistance genes so far described are probably either involved in signal perception and/or signal transduction. For most of these genes, the function has yet to be proven formally, with the further exception of *Pto* (see below). The LRR motif is a constant theme in the structure of the gene products and is likely to be involved in perception of the avirulence signal. The nature of the avirulence signal will determine the location of the LRR. For instance, the LRR will be extracellular for *Cf-2* and *Cf-9* as the pathogen ramifies among the intercellular spaces of the host and the active product can be extracted from those spaces, whereas the N gene product is likely to detect the TMV avirulence signal intracellularly. One of the surprises of resistance gene analysis is the likelihood that the products of several genes are located in the cytoplasm, either suggesting that the pathogen is only detected after cellular penetration or that the avirulence signal can enter the cell. In the case of *Pto*, although located in the cytoplasm, it could be associated with another protein, possibly containing an LRR, that may or may not span the cell membrane. It is important to note that plants apparently do not distinguish between different forms of pathogen in terms of detection and use the LRR structure to sense any invader.

Once the presence of a pathogen has been detected, it is essential for the plant to initiate a response. Consequently, most resistance genes have structures which imply that they are involved in signal transduction. Three specific features have been resolved, namely, serine/threonine kinases, nucleotide-binding sites and Toll/IL-1R homologies. Whether these structures feed into the same or different signal transduction pathways is a major question for future research (see Schulze-Lefert *et al.*, Chapter 3 this volume).

How Do Plants Use Disease Resistance Genes to Detect a Wide Range of Possible Pathogens?

Plants need to be able to detect invasion by a wide range of potential pathogens and, hence, must be capable of generating variation in the detection capability present in the genome. The genomic organization of loci for resistance genes and the molecular structure of the genes themselves provide clues to how this may be achieved by plants.

Gene duplication

When some of the cloned resistance genes have been used as probes to Southern blots of genomic DNA from the plants from which they were cloned, they have typically revealed the presence of additional copies of similar genes.

When the L^6 gene was used as a probe to a cDNA library at least five different classes of cDNA were identified (Ellis *et al.*, 1995). Only one of these mapped to the L^6 locus whereas the remainder mapped to the unlinked and genetically complex locus M . This suggests that L and M flax rust resistance genes are similar and may have arisen by duplication and translocation. Different forms of the L locus in different plants appear to be allelic whereas there are several resistance determinants in the M locus. It would appear that gene duplication and then mutation has occurred at the M locus to produce new resistance specificities whereas only mutation has occurred at the L locus. The repetitive nature of the LRR structures within these genes may make them inherently unstable and prone to intragenic rearrangements, resulting in new detection capabilities.

In the case of Cf genes of tomato, a great deal of DNA sequence level and protein structural homology is seen between the Cf -2 and Cf -9 genes (Dixon *et al.*, 1996). Both genes are part of multigene families, each member of which potentially has pathogen recognition capability. Most interestingly, two nearly identical genes (they only differ by three nucleotides) are present at the Cf -2 locus, both of which can function on their own to recognize the presence of *C. fulvum* carrying *Avr2*. These genes presumably arose by a recent DNA

duplication event. Further mutation could generate the ability to detect new pathogen isolates. Hence, new resistance specificities can be generated by duplicating genes and altering their primary structure.

A further twist to the advantages of gene duplication was revealed at the *Pto* locus. *Pto* is part of a complex locus containing five to seven genes that are all serine/threonine kinases and highly similar to *Pto*. It had been observed previously that plants carrying the *Pto* gene were sensitive to the application of the organophosphate insecticide fenthion and produced small necrotic lesions, similar to those produced in the hypersensitive response. Analysis of the *Pto* locus showed that one of the other kinases (*Fen*), and not *Pto* itself, was responsible for the sensitivity to fenthion (Martin *et al.*, 1994; Salmeron *et al.*, 1994). Hence, the genes *Pto* and *Fen* produce a similar plant response on exposure to very different stimuli, a plant pathogen and a synthetic chemical, respectively. This shows that the detection mechanisms of plants are not limited to proteins and allow the plant to respond to secondary metabolites produced by the pathogen.

Keen *et al.* (1990) and Midland *et al.* (1993) showed that the active molecule produced by *avrD* from *P. syringae* pv. *glycinae* was a small molecule similar to a syringolide, which would be consistent with the detection capability of *Fen*. Interestingly, *Pto*, when it is overexpressed in tomato plants, confers a mild sensitivity to fenthion (Martin *et al.*, 1994), suggesting that the genes may respond to similar signal molecules. Although the ability of *Fen* to respond to fenthion may only be a consequence of a chance similarity of the insecticide to a naturally occurring compound, or a redundant detection capability of the *Pto* locus, this none the less demonstrates the advantages of gene duplication in increasing the range of molecules that the plant can detect.

Another advantage of duplicating gene sequences at the same locus is the increased potential for intergenic recombination, resulting in the generation of new forms of resistance genes. This is demonstrated most clearly in the work on the *Rp1* locus of maize. At least 14 different specificities that confer resistance to *Puccinia sorghi* (yellow rust) map to the *Rp1* locus (Hooker and Saxena, 1971). Analysis of this region with molecular markers suggests that it is highly unstable and loss of gene function can be detected. This has been attributed to unequal crossing over between tandemly repeated elements across the locus (Bennetzen *et al.*, 1988; see Hulbert *et al.*, Chapter 2 this volume). Intergenic recombination could not only lead to loss of function but also to the generation of new resistance specificities.

Studies of the interaction between *Peronospora parasitica* (downy mildew) and *Arabidopsis* have revealed the existence of numerous recognition specificities (Holub and Beynon, 1997; see Holub, Chapter 1 this volume). Resistance genes appear to be present on all five *Arabidopsis* chromosomes and in several cases these fall into regions each covering approximately 15 cM. This implies that large regions of *Arabidopsis* chromosomes are involved in specifying disease resistance and the clustering could imply evolution by duplication and

rearrangement. However, cloning of these loci is still necessary to reveal the nature of the genome at these locations. Other parasites, notably *Erysiphe* (powdery mildew) genes seem to map elsewhere in the *Arabidopsis* genome (Kunkel, 1996; see Holub, Chapter 1 this volume). This increases the percentage of the genome involved in detecting pathogens.

Hence, gene duplication, mutation and recombination can produce a range of resistance specificities. However, this would still not explain the vast range of resistance capability exhibited by plants. The structure of the resistance genes reveals new clues as to ways in which plants can increase this detection capability without large numbers of new genes.

Cooperative gene function

A single resistance gene product can only have a limited range of detection capability. However, this could be greatly enhanced if more than one gene could interact to detect novel signals. There is, as yet, no proof that this occurs in disease resistance but the structures shown by the resistance genes analysed to date suggest that such cooperation is likely.

The increasingly complex story of *Pto* and its associated genes provides the best understood example. Salmeron *et al.* (1994) showed that a third gene, *Prf*, closely linked to *Pto* and *Fen*, was required for recognition of both *avrPto* and fenthion. Recently, the structure of *Prf* has been determined and shown to contain an LRR structure and a nucleotide-binding site (Salmeron *et al.*, 1996). Additionally, Zhou *et al.* (1995) have isolated a gene, *Pti*, that is specifically phosphorylated by *Pto*. *Pti* is another serine/threonine kinase which is probably cytoplasmic as, unlike *Pto*, it does not contain a potential myristoylation sequence. *Pti* is not phosphorylated by *Fen* and cannot phosphorylate *Pto*. Given that *Prf* contains an LRR structure, as do all other resistance genes cloned to date, and that *Pti* is downstream of *Pto*, it is likely that *Prf* is involved in the direct detection of a signal molecule. When *avrPto* or fenthion are detected via *Prf*, corresponding kinases *Pto* or *Fen* become phosphorylated and pass the specific phosphorylation signal on to different pathway intermediates. Hence, by using a single LRR gene, two different signals may be detected. The gene cluster containing *Pto* and *Fen* includes several other kinases all of which may interact with *Prf* to detect other signals. There is no reason why these kinases should not interact with other LRR genes to detect other signals. This would increase greatly the number of pathogens that a small number of genes could detect.

Tomato plants lacking resistance to *C. fulvum*, *Cf-O*, bind the *Avr9* protein product (Jones *et al.*, 1994). However, these plants will only express resistance to the pathogen when transformed with the *Cf-9* gene. This is initially a surprising result since the LRR nature of *Cf-9* would imply direct interaction with the *Avr9* gene product. Perhaps the *Avr9* gene product is bound by one or more

LRR gene products, although among these only *Cf-9* is capable of transmitting a signal, or else the binding by other genes enhances the ability of *Cf-9* to respond to the pathogen, either by direct or indirect (via the bound *Avr* signal) interaction. In this way, the potential of a limited number of LRR-containing genes to detect a range of signal molecules could be increased.

The leucine zipper structures of *Rpm1* and *Rps2* (Bent *et al.*, 1994; Mindrinos *et al.*, 1994; Grant *et al.*, 1995) suggest that protein dimerization may play a role in generating variation in the detection capabilities of resistance genes. Leucine zippers are used to allow proteins containing them to form dimers, therefore, it is possible that several such proteins carrying different LRR structures can combine in a variety of dimeric forms, each with its own detection capability. However, both *Rpm1* and *Rps2* are not part of gene clusters, so the feasibility of such a system has yet to be proven.

Multiple recognition specificity

There is no reason why a particular resistance gene should only recognize a single *avr* gene signal or, equally, that the *avr* signal should only be detected by one resistance gene. Bisgrove *et al.* (1994) showed that mutations in the *Rpm1* gene of *Arabidopsis* resulted in the loss of recognition of both *avrB* and *avrRpm1*. The protein products from these *avr* genes are apparently unrelated (Tamaki *et al.*, 1988; Dangl *et al.*, 1992), although it must remain a possibility that they are involved in the production of a similar signal molecule, suggesting that *Rpm1* is capable of recognizing more than one signal molecule. The mutation in *Prf* (Salmeron *et al.*, 1994) also suggests that this gene detects more than one *avr* gene product (in addition to fenthion), as an avirulent *P. syringae* strain lacking *avrPto* becomes virulent on plants carrying the mutation. Furthermore, functional homologues of *Rpm1* exist in pea, bean and soybean (Dangl *et al.*, 1992), implying that genes in these plants, which are essentially unrelated to *Arabidopsis*, have evolved the ability to detect the same *avr* gene signal. If different resistance genes can exist in different plants, there is no reason why they should not occur in the same plant. Indeed, *Cf-2*-containing plants carry two different genes both capable of detecting the same *avr* gene signal.

A summary of *avr* gene signal recognition capability

From the analysis of resistance gene structure, it is clear that an immense potential exists in plants for generating novel detection capability. This variability can arise from intragenic rearrangements based on the repetitive nature of the LRR structures in addition to normal rates of mutation. This variation is enhanced further by the duplication of resistance genes and the potential for

intergenic recombination resulting in novel forms of gene product. The variability is potentially multiplied many fold by genes working together, be they two LRR-containing genes or LRR genes working in concert with a series of protein kinases. With these insights, the ways in which plants manage to be resistant to so many potential pathogens is becoming understood more clearly.

A Functional Model for Disease Resistance in Plants

A model for resistance gene function is illustrated in Fig. 19.3. Leucine-rich repeat molecules are probably the primary gene products that interact with the *avr* gene signal molecules. The LRR structures can be extra- or intracellular, possibly suggesting different locations of the *avr* gene product that is detected. These gene products are potentially capable of recognizing multiple *avr* gene signal molecules. The genes with extracellular LRR domains detect extracellular AVR signal molecules and are anchored to the plant cell membrane.

In situation 1 of Fig. 19.3, a gene like *Cf-9* binds to the AVR signal and then transfers this signal either by interacting directly with an NADPH oxidase, resulting in an oxidative burst, or interacting with a further LRR gene (situation 1a) that does have an associated signal transduction capability (e.g. *Xa21I*), or interacting with a membrane associated protein kinase (e.g. *pto*).

Situation 2 represents genes such as *Xa21* where an extracellular LRR is attached to an intracellular protein kinase via a membrane-spanning domain. Such proteins are potentially capable of detecting the *avr* gene signal and transmitting that detection to a cellular signalling cascade resulting in disease resistance. The kinase function could then phosphorylate another kinase and the particular protein/protein interaction could be determined by the *avr* signal molecule detected. This structure potentially has great flexibility in that it could associate with any number of other LRR-containing molecules in order to detect a range of *avr* gene signal molecules.

Situation 3 would be similar to that of *Xa21* except that no direct kinase function is attributable to the LRR-containing molecule. However, such a protein could associate with other LRR proteins and interact with a range of kinases enabling the plant to respond to *avr* gene signal molecules.

The LRR proteins can also be located intracellularly and a range of associations with other proteins and variability, similar to that found in the membrane-spanning proteins, can be postulated. Situation 4 would be similar to that of the *N*, *RPS2* and *RPM1* genes where an intracellular LRR detects an *avr* gene signal and transmits that information via a signal transduction pathway to disease response genes. The *N* and *L⁶* genes also contain sequences similar to those of Toll and IL-1 proteins, which may imply a role in the direct activation of transcription factors resulting in a disease resistance response. This detection capability may be enhanced by the formation of dimeric molecules via leucine zippers, which possibly are present in *RPS2* and *RPM1* (situation 5).

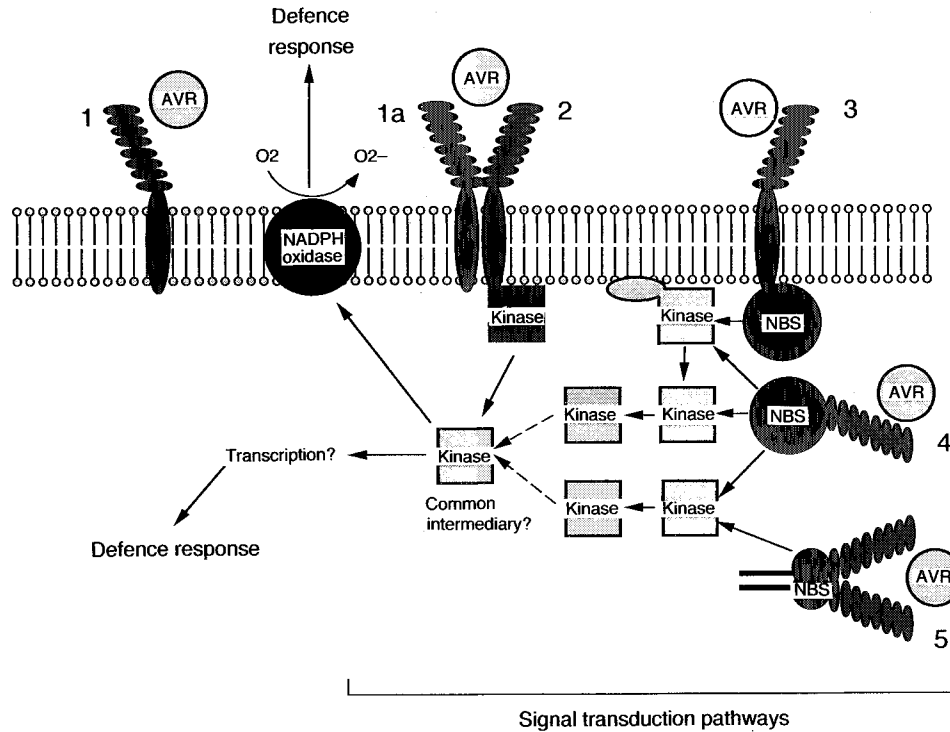


Fig. 19.3. Models for resistance gene function (see text for further explanation). AVR = the active product of the *avr* gene; NBS = nucleotide binding site.

Once the presence of an *avr* gene signal is perceived, that information is transmitted to a disease response mechanism. The nature of this pathway is still unknown but clues are beginning to be revealed. From the work on *pto* (Dangl *et al.*, 1992; Martin *et al.*, 1993, 1994; Salmeron *et al.*, 1994; Innes, 1995) it is clear that kinases can play an important role in the signal transduction pathway. The ability of one LRR gene product to interact with more than one kinase in an *avr* gene product-specific manner reveals a vast range of potential variability in signal detection. One kinase could interact with several LRR gene products or with only one. That signal could be transmitted directly to cellular response mechanisms or to another kinase. Each kinase pathway could be unique or disease resistance pathways could flow through common intermediate steps.

Finally, all this capability for signal detection results in a disease resistance response possibly via gene transcription or directly via an oxidative burst. Zhou *et al.* (1995) reported that some proteins which interact with the *Pto* product show similarity to transcription factors and such transcription factors can be activated by phosphorylation (Hunter and Karin, 1992). Hence, phosphorylation of *Pto* may lead to transcriptional activation of several genes involved in the disease resistance response, such as chalcone synthase, phenylalanine ammonium-lyase and pathogenesis-related proteins (Cutt and Klessig, 1992; Greenberg *et al.*, 1994). Levine *et al.* (1994) showed that protein phosphorylation is required for the induction of the oxidative burst in an interaction between soybean and an incompatible isolate of *P. syringae* pv. *glycinea*. Zhou *et al.* (1995) suggested that *Pti* may phosphorylate a protein homologous to the human p47 which is then relocated to activate the plasma membrane NADPH oxidase and, hence, results in an oxidative burst (Babior, 1992).

This model is speculative but is, none the less, based on clues that were revealed by the cloning and analyses of only a few resistance genes. It suggests a complex and highly variable capability to detect the presence of pathogens and respond to their invasion. Interestingly, studies utilizing mutation to reveal steps in signalling pathways have resulted in the discovery of only a few additional genes that are required for the function of the naturally variable 'resistance gene'. Examples of such studies are the *Rcr-1* and *Rcr-2* genes from tomato, which are required for *Cf-9* function (Hammond-Kosack *et al.*, 1994), and *Nar-1* and *Nar-2*, which are necessary for powdery mildew resistance in barley mediated by the *Mla12* gene (Freialdenhoven *et al.*, 1994; Schulze-Lefert *et al.*, Chapter 3 this volume). The small number of mutant classes has two potential explanations. There are possibly very few steps from the initial detection of the *avr* gene signal molecule to the resistance response. Alternatively, the genes involved in the signal transduction pathways that are unique to disease resistance are few, but they in turn feed into pathways that are essential to cell survival and, hence, mutants cannot be identified. This second route is fascinating because it suggests the possibility that several different

stress responses could be mediated via convergent signal transduction pathways.

Recently, a gene encoding a metallothioneine-like protein that is specifically expressed during leaf senescence in *Brassica* has been cloned (Buchanan-Wollaston, personal communication). When the promoter of this gene is linked to the β -glucuronidase reporter gene (*uidA*) and transformed into *Arabidopsis*, it is found to be expressed during leaf senescence. However, this promoter is also activated in the presence of the fungal pathogen, *P. parasitica*, but only by isolates to which the host plant carries a resistance gene. No transcription is detected when the plant is inoculated with a compatible isolate (Butt and Buchanan-Wollaston, personal communication). This suggests that the signal transduction pathways involved in pathogen detection and senescence can result in the transcription of the same genes. Therefore, the signalling pathways overlap at some point, possibly at an early stage, hence the paucity of disease resistance-specific mutants, or at the level of activating specific transcription factors. If the pathways to the disease resistance and senescence responses overlap, it is possible that other stress-related responses (e.g. wounding) are also mediated via overlapping mechanisms. The analysis of such pathways is an important area of current and future research not only for disease resistance but for many areas of plant biology (Nasrallah *et al.*, 1994; Ecker *et al.*, 1995).

Is the Gene-for-Gene Concept Still Valid?

The gene-for-gene model was the product of studies utilizing natural variation within plant species. Hence, susceptible plants were compared with resistant ones and genes specifically involved in the interaction with the *avr* gene products defined. As a description of the variation between any two plants, the gene-for-gene model is still valid. However, it is now clear that disease resistance commonly involves more than one gene, clearly demonstrated in *pto*-mediated resistance. Furthermore, the definition of the 'resistance gene' will be dependent on the natural variation between any two plants. For example, in the *pto* resistance pathway, natural variation was detected that led to the cloning of the distinct kinases, *pto* and *fen*, accountable for the response to two different stimuli. However, if the natural variation had been at the level of *prf*, then a single LRR-containing gene would have been cloned, the presence of which would lead to a plant response to both the *avrPto* signal and fenthion. As a consequence, very different 'resistance genes' could have been cloned, all of which would have been the single gene defined under the gene-for-gene concept.

It is now appropriate to move beyond the strict gene-for-gene concept and instead view disease resistance as a process that results from several gene products working in concert. These would include: genes involved in pathogen

detection (currently LRR-containing proteins); genes that are part of signal transduction pathways (possibly including, but not limited to, kinases and phosphatases and transcription factors); and genes that are involved in the disease resistance response (either as transcribed products or via the oxidative burst).

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Elicitor Generation and Receipt – the Mail Gets Through, But How?

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The recent cloning of plant disease resistance genes and the isolation of avirulence gene-specified elicitors will fuel an explosion in our understanding of natural disease defence mechanisms in plants, much as the discovery of immunoglobulin gene rearrangement did for vertebrates in the 1980s. In this chapter I will review recent developments concerning the generation of elicitor signals in pathogens and their perception by plants carrying the cognate disease resistance genes. Considerable progress has occurred in our understanding of plant disease resistance in recent years, and it is clear that the generation of elicitors by pathogens as well as elicitor perception by resistant plants are more complex than originally envisioned. I will discuss some of these developments; see also Chapter 19 in this volume by Beynon. I will refer to local resistance mechanisms collectively under the historic term 'hypersensitive reaction' (HR).

As known since the beginning of this century, some cultivars of a plant species may recognize a particular pathogen and invoke the HR, while susceptible cultivars do not. The resistant plants are generally found to harbour single Mendelian plant disease resistance genes and these have been a mainstay of disease control in agriculture. However, pathogen strains frequently emerge which 'overcome' resistance genes and cause disease. Such strains generally exhibit mutations in avirulence genes and consequently fail to produce signal molecules, called specific elicitors. Strains carrying a functional avirulence gene produce the corresponding elicitor, but it functions only in plants carrying the complementary resistance gene. Since they mimic the specificity of the pathogen, specific elicitors are the equivalent of antigens in vertebrate pathogens.

Elicitors and Their Receptors

Two types of elicitors are known: general elicitors, which do not exhibit differences in cultivar sensitivity within a plant species; and specific elicitors, which function only in cultivars carrying matching disease resistance genes (for review, see Boller, 1995). General elicitors include substances associated with basic pathogen metabolism, such as cell wall glucans, chitin oligomers and glycopeptides, while specific elicitors usually have more unique structures (proteins, peptides and the syringolides to be discussed later) and their production only occurs as a consequence of avirulence gene function.

The isolation of avirulence gene-specific elicitors strongly supports the elicitor-receptor hypothesis, which states that pathogen avirulence genes specify production of specific elicitors which are, in turn, perceived by receptors in the resistant plant. Substantial evidence also exists for the occurrence of plant receptors which recognize general elicitors (see Boller, 1995). However, perception of avirulence gene-specific elicitors by resistant plants may be more complex than their differential binding by resistant but not susceptible plants. In two cases, labelled specific elicitors have been shown to bind with similar affinity to plant extracts, regardless of their resistance genotype. In the case of the *avr9* peptide elicitor from *Cladosporium fulvum*, Honée *et al.* (1994) observed saturable, ligand displaceable binding to plasma membrane preparations from either *Cf9* or *cf9* tomato genotypes. Labelled syringolides, produced by bacteria expressing avirulence gene *avrD* also bound specifically to a site in the soluble fraction of soybean leaves, but no difference was observed in the binding to extracts from soybean cultivars containing or lacking the cognate disease resistance gene, *Rpg4* (Y. Okinaka, Y. Takeuchi, N. Yamaoka, M. Yoshikawa, C. Ji and N. Keen, manuscript in preparation). These results raise the possibility that resistance gene products may not be directly involved with elicitor binding, but instead may be components of signal transduction pathways leading to defence response gene activation. The structure of certain resistance gene proteins, most notably *Pto* (Martin *et al.*, 1993), argues that they are probably involved in signal transduction rather than elicitor perception, *per se*. If this is the case, what are the elicitor binding sites and how is specificity accounted for?

Plant Disease Resistance Genes and Pathogen Avirulence Genes

Plant disease resistance genes recently have been cloned and characterized from several plants (for recent reviews see Michelmore, 1995; Staskawicz *et al.*, 1995; Martin, 1996; Beynon, Chapter 19 this volume). These genes encode proteins that fall into three general classes: (i) proteins with protein kinase

domains and possible membrane-anchoring myristylation domains such as the tomato *Pto* gene product (Martin *et al.*, 1993); (ii) proteins with leucine-rich repeat (LRR) domains, P-loops and possible transmembrane-spanning domains, such as the tomato *Cf9* gene product (e.g. Jones *et al.*, 1994); (iii) a hybrid with LRR, leucine zipper and protein kinase domains all in the same protein, exemplified by the rice *Xa-21* gene product (Song *et al.*, 1995). While their biochemical functions are not yet established, it is possible that the LRR domains are involved in elicitor recognition and may activate the kinase or P-domains to initiate signal cascades eventually resulting in the activation of defence response genes (Dangl, 1995). An important requirement to test this idea critically is the availability of cognate avirulence gene-specified elicitors, but only a few have so far been isolated.

More than 40 pathogen avirulence genes have been cloned and characterized (Long and Staskawicz, 1993). Unlike plant disease resistance genes, they do not resemble known genes in the databases. Certain fungal primary avirulence gene products are processed and secreted extracellularly, where they function as elicitors (see de Wit, 1992). Bacterial avirulence gene proteins have not been shown to be secreted, fostering the speculation that they may have enzymatic functions inside the cell. This proposition is supported by the syringolide elicitors directed by *avrD*, discussed later. However, recent work indicates that members of the *avrBs3* family possess functional plant nuclear targeting sequences (Yang and Gabriel, 1995). While these sequences have not yet been shown to direct avirulence gene proteins to the plant nucleus, this is none the less an appealing possibility.

***Pseudomonas syringae* Avirulence Genes and Their Relationship to *hrp* Genes**

Although at least ten different avirulence genes have been cloned from members of the *P. syringae* group, only one has been demonstrated to direct production of an isolable elicitor following expression in *Escherichia coli* or other bacteria. This gene, called *avrD*, directs the production of unusual acyl glycosides, called syringolides, when expressed in *E. coli* or several other Gram-negative bacteria (Keen *et al.*, 1990; Midland *et al.*, 1993). Bacteria expressing *avrD* or the purified syringolides specifically elicit the HR in soybean cultivars carrying the *Rpg4* disease resistance gene. The syringolides are of particular interest because their amphipathic properties permit ready egress from bacterial cells, much like the well known acyl homoserine lactone autoinducers of *Vibrio sp.*, *Pseudomonas aeruginosa* and other bacteria (see Winson *et al.*, 1995). Unlike *avrD*, other cloned *P. syringae* avirulence genes, including *avrA*, *avrB*, *avrC*, *avrE*, *avrRpm1*, *avrRpt2*, *avrPph3* and *avrPto* do not enable *E. coli* cells to cause the HR in predicted plant cultivars or to produce isolable elicitors.

Lindgren *et al.* (1986) discovered a large (about 22 kb) chromosomal gene cluster in *P. syringae* pv. *phaseolicola* that was necessary for pathogenesis on bean plants and also required for formation of the HR on non-host plants such as tobacco. This cluster contained the *hrp* (hypersensitive response and pathogenicity) genes (for review, see Willis *et al.*, 1991). Research in several laboratories has contributed to the characterization of *hrp* genes in *P. syringae* pathovars and other plant pathogenic bacteria. These clusters contain several transcriptional units with one or more cistrons, many of them encoding components of a type III extracellular secretion system that is conserved in several bacterial pathogens of vertebrates, such as *Salmonella* and *Yersinia* (Salmond, 1994). The precise functions of the various *hrp* gene products in secretion and identification of the secreted molecules are topics of active research in several laboratories studying plant and vertebrate pathogens.

The laboratory of Steven Beer discovered that one gene in the *Erwinia amylovora* *hrp* gene cluster encodes an unusual protein, called harpin, which elicits the HR in non-host plants (Wei *et al.*, 1992). Harpins have been discovered in other pathogenic bacteria and are unusual in being stable to boiling and relatively glycine rich, but have hydrophobic domains and exhibit surfactant properties, indicating that they may interact with cell membranes. Harpins may be highly evolved virulence factors tailored to particular host plant species, the penalty being that such a customized harpin protein may non-specifically elicit the HR in other plants.

The *P. syringae* *hrp* genes and ten different avirulence genes have a common promoter (see Xiao and Hutcheson, 1994), called the '*avr/hrp* box' (GGAACC-N 15/16-CCAC). The coregulation of *hrp* and *avr* genes in these bacteria suggested that they may also be functionally related and encouraged the idea that the function of certain avirulence genes may require the *hrp* gene cluster. Indeed, Pirhonen *et al.* (1996) recently showed that *E. coli* MC4100 or *P. fluorescens* cells carrying the cloned *P. syringae* pv. *syringae* *hrp* gene cluster in addition to any one of seven different cloned *P. syringae* *avr* genes (*avrA*, *avrB*, *avrC*, *avrPph3*, *avrRpt2*, *avrRpm1* or *avrPto*) elicited the HR only in soybean, tomato or *Arabidopsis* cultivars carrying the complementary disease resistance genes. *E. coli* or *P. fluorescens* cells expressing only the various cloned avirulence genes elicited no detectable reaction, demonstrating the necessity of the *hrp* gene cluster. HR competence was shown genetically to require the *hrp* secretion genes, but deletion analysis indicated that harpin, encoded by the *hrpZ2* gene, was not essential. Consistent with previous work, *E. coli* cells expressing *avrD* produced the expected syringolides independently of the *hrp* gene cluster. These results are of considerable significance because they suggest that, with the exception of *avrD*, the secretion functions of the *hrp* genes may be required to deliver *P. syringae* avirulence gene proteins to or into the plant cell.

LRR Proteins and the Parallel β -Helix

The LRR domains of *Cf9* and other LRR resistance gene proteins are fascinating since their repeat nature is suggestive of the unique structural fold discovered by Marilyn Yoder, Frances Journak and ourselves in pectate lyase enzymes (Yoder *et al.*, 1993). These proteins were shown to have a totally new and revolutionary structure, called the parallel β -helix, consisting of a right-handed helix with a minimum of 22 amino acids per turn in the β configuration with stacks of identical or similar amino acids located in a ladder array on the helix. These stacks of asparagines, hydrophobic residues and aromatic residues stabilize the helical structure by hydrogen or hydrophobic bonding. After description of the parallel β -helix, several other proteins were subsequently shown to have the same fold. In addition, Raetz and Roderick (1995) also recently reported that a bacterial acyltransferase protein possessed a subunit structure based on a left-handed parallel β -helix.

LRR proteins are thought to be involved in protein-protein interactions (Kobe and Deisenhofer, 1994). However, the only LRR protein with a known X-ray structure is porcine ribonuclease inhibitor. The protein has LRRs of 28 or 29 amino acids organized into β - β structural units with a parallel β -sheet surface exposed to solvent. This gives the protein a horseshoe shape which is assumed to facilitate protein-protein interactions (Kobe and Deisenhofer, 1994). However, as these authors note, there is no reason that certain LRR proteins might not alternatively assume the parallel β -helix structure of the pectate lyases. Indeed, sequence prediction studies as well as biophysical characterization by FT-IR (Fourier-transform infrared spectroscopy) and CD (cluster of differentiation) measurements (Sieber *et al.*, 1995) suggest that proteins with LRR repeats of 25 or fewer amino acids are most likely folded into a parallel β -helix rather than the β - β structure of porcine ribonuclease inhibitor (Yoder and Journak, 1995).

Defence Responses

Once plant defence is initiated, intracellular signalling cascades ultimately result in the transcriptional activation of batteries of genes called defence response genes (Dixon and Harrison, 1994; Godiard *et al.*, 1994). These encode a diverse array of proteins, including those required for the production of phytoalexins, cell wall re-enforcing proteins, and proteins that are directly antagonistic to pathogens. Biochemical and genetic work has identified several putative components of the intracellular signalling pathway connecting elicitor recognition and defence gene activation in plants. Table 20.1 summarizes some of these putative signal transduction components, but it is not yet possible to organize them into a coherent model.

Table 20.1. Some putative components of intracellular signalling pathways involved in the induction of defence response genes in plants.

Component	Identified by	Reference
<i>Pto</i>	Cloning and expression	Martin <i>et al.</i> , 1993
<i>Prf</i>	Mutation	Salmeron <i>et al.</i> , 1994
<i>Pti</i>	Required for <i>Pto</i> activity	Zhou <i>et al.</i> , 1995
<i>nim1</i>	Mutation	Delaney <i>et al.</i> , 1995
Salicylic acid	<i>nahG</i> transgenic plants	Delaney <i>et al.</i> , 1994
Jasmonic acid	Biochemical work	Gundlach <i>et al.</i> , 1992
Calcium uptake	Biochemical work	Tavernier <i>et al.</i> , 1995
Protein phosphorylation	Biochemical work	Chandra and Low, 1995
Phosphatase inhibition	Biochemical work	Boller, 1995
Extracellular alkalization	Biochemical work	Boller, 1995
Active oxygen species	Biochemical work	Vera-Estrella <i>et al.</i> , 1993
<i>Rcr-1/Rcr-2</i>	Mutation	Hammond-Kosack <i>et al.</i> , 1994
<i>Ndr1</i>	Mutation	Century <i>et al.</i> , 1995
<i>Nar-1/Nar-2</i>	Mutation	Freialdenhoven <i>et al.</i> , 1994
Transcription factors	Biochemical work	Dixon and Harrison, 1994

A Great Deal Remains to be Done

Several pieces are in hand but not properly placed to complete the jigsaw puzzle that is active disease resistance in plants. To rephrase the title, we only partially understand events occurring between pathogen avirulence gene function and ultimate activation of defence response genes in the resistant plant host. Further study of this mail delivery route will interest scientists well into the next century because improved disease control is the prospective gain. For example, Rommens *et al.* (1995) and Thilmony *et al.* (1995) have already reported that the *Pto* resistance gene from tomato functions when transformed into tobacco plants. Several aspects of disease resistance remain to be explored: what is the nature of the intriguing salicylic acid link between the HR and systemic acquired resistance (Delaney *et al.*, 1994)? Do bacterial *hrp* secretion proteins deliver avirulence gene proteins to the plant cell? Will all plant species possess similar perception and signal transduction cascades? Also, since disease resistance is a tissue response and not solely a cellular response (e.g. Graham and Graham, 1994), does greater complexity occur in cell-to-cell signalling events? Finally, little attention has been paid to the question of signal damping in resistant plants. In addition to perception and response to pathogen elicitors, plants must also be equipped with devices to sequester or degrade elicitor molecules in order to prevent a permanent 'on' situation.

The availability of cloned plant disease resistance genes should permit biochemical testing of the as yet unproven idea that the LRR domains directly interact with cognate specific elicitors. Furthermore, the availability of these

elicitors will accelerate biochemical and genetic approaches to identify members of signal transduction pathways. Because of the great power of X-ray crystallography and other approaches to determining protein structure, we can also expect progress in understanding ligand perception and signal transduction at the atomic level. There will also be considerable interest in the construction of chimeric disease resistance genes in which the LRR domains have been altered either by *in vitro* recombination or the introduction of defined mutations. Will it be possible in this way to target novel features of pathogens as elicitors, such as pectic enzymes, toxins or other virulence factors?

The rates at which various resistance genes activate defence response genes also vary as exemplified by resistance genes that confer temporally different phenotypic expression of resistance in the same genetic background. Colloquially, one could use the terms 'fast' and 'slow' resistance genes to identify the extremes. They may involve variable efficiencies of elicitor perception or differences in the signal transduction elements slaved to particular resistance genes, but may also reflect the dynamics of elicitor delivery.

How pathogens deliver elicitors to plant cells is a relatively unstudied area. The *Cladosporium fulvum* peptide elicitors require processing by fungal and/or plant enzymes to generate the final, elicitor-active molecules (van den Ackerveken *et al.*, 1993). The rate at which these reactions occur will temporally influence the ultimate plant resistance phenotype, particularly in environmental situations where elicitor processing varies. The syringolide elicitors, which are formed by the pathogen via an enzymatic mechanism, may also exhibit marked temporal differences in production, depending on the environment. *P. syringae* pv. *glycinea* cells expressing *avrD* cause a visible HR in *Rpg4* soybean plants only after approximately 36 h at 22°C. Infiltration of purified preparations of the syringolides into *Rpg4* soybean leaves, however, causes a visible HR after 10 to 24 h, depending on the concentration. The delayed HR in bacteria-inoculated leaves presumably must reflect the dynamics of *hrp* gene activation in the pathogen and expression of the *avrD hrp/avr* promoter, followed by the time required to synthesize the AvrD protein and to produce and secrete sufficient concentrations of the syringolides in the leaf intercellular space to activate *Rpg4*-mediated resistance.

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Learning from the Mammalian Immune System in the Wake of the *R*-Gene Flood

21

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The recent cloning of plant disease resistance (*R*) genes directed against several classes of plant pathogens and isolated from a variety of species has focused our attentions on how the encoded proteins recognize pathogen-derived avirulence (*avr*) gene signals, how that recognition is transmitted by the plant cell into an effective resistance response and how new *R*-specificities evolve. It may be useful to use the animal immune system as a more advanced paradigm for understanding the genetic organization and biochemical mechanisms of the response networks of plant disease resistance.

Themes

Plant disease resistance loci have several intriguing genetic features in common with the animal immune system, particularly that arm of the immune system responsible for displaying pieces of 'foreign' and 'self' peptide antigens to the effector arm of cellular immunity. A second aspect of mammalian immunity, namely innate immunity, also has certain parallels with plant disease resistance, especially with respect to its use of an oxidative burst to help destroy intracellular parasites. The use of recombinant inbred mice has also revealed that innate mammalian immune responses can be dictated by allelic differences at single loci not unlike the gene-for-gene response (Hoffman, 1995; McLeod *et al.*, 1995; Fearon and Locksley, 1996). This aspect can also serve as a paradigm for comparison with biochemical diversity in plant *R*-gene function.

I will introduce several themes, focusing on recognition diversity generated by the mammalian major histocompatibility complex (MHC), and return to each in turn. First, the clustering of genetically defined resistance specificities into large complexes, suggests analogies to multigenic arrays of MHC genes (Dangl, 1992b). One obvious implication is that this clustering provides the raw material for evolution of new disease resistance specificities in multigene families. Second, this clustering of resistance specificities could be due to multiple biochemical functions being encoded in a linked region, also as observed in the MHC. This gives rise to the notion of the 'haplotype'; evolutionary maintenance of linked genes encoding proteins which act together in a common system. Third, and following from the second, is the idea that there are several steps in the disease resistance pathway at which polymorphism could be selected positively as providing a more effective end result. This allows more mixing and matching of recognition and signalling functions until very intricate pathways are created. Such interweaving of response is a hallmark of eukaryotic signal transduction (Cooper, 1994) and may be advantageous for quick, flexible adaptation to new pathogen threats.

Clusters and the Diversity Conundrum

Genetic analyses of gene-for-gene recognition of pathogens in a variety of systems revealed long ago a predilection for clustering of different *R*-gene specificities at a given locus. There seem to be two major variants. The first is an array of different specificities, each recognizing a particular isolate of pathogen (and presumably a particular *avr* function). These specificities can be linked in *cis* genetically. The classic examples of this are the maize *Rp1* locus (Ullstrup, 1965; Saxena and Hooker, 1968; Pryor, 1987a, 1987b; Bennetzen and Hulbert, 1992) and the flax *M* locus (Mayo and Shepherd, 1980). In contrast, the second paradigmatic *R*-locus type is exemplified by the flax *L* locus, where many alleles encoding different specificities exist, but where no more than one can be brought on to the same chromosome in *cis* (Flor, 1965; Shepherd and Mayo, 1972; Islam *et al.*, 1989; Islam and Shepherd, 1991). The advent of molecular mapping has made possible dissection of the chromosomal events at these loci which are responsible for *R*-gene clusters. For *Rp1*, it has been shown that both unequal crossover between sister chromatids and gene conversion have roles to play (Bennetzen *et al.*, 1988; Sudupak *et al.*, 1993; Hu and Hulbert, 1994). Fulfilling one prediction of the idea that clusters exist to promote the evolution of novel *R*-specificities, it was shown that new *Rp1* alleles are created during these recombination events (for more on this topic, see Richter *et al.*, 1995 and Hulbert *et al.*, Chapter 2 this volume).

Molecular cloning of *R*-genes in several systems (reviewed by Dangl, 1995; Staskawicz *et al.*, 1995; Boyes *et al.*, 1996; Beynon, Chapter 19, this volume) should make possible an ever more detailed appraisal of the events that drive

R-gene evolution. A key example of the impact of cloning of R-genes on understanding of how R-loci evolve and new specificities emerge will come from the dissection of the flax *L* and *M* loci. The many specificities at *L* are truly allelic and large efforts have failed to find *cis* combinations of two specificities (beautifully reviewed in Lawrence *et al.*, 1994). The cloning of *L6* (Lawrence *et al.*, 1995) will allow rapid molecular analysis of the other *L* alleles, and will surely contribute to an understanding of the structural correlates of R-gene specificity. The fascinating finding that a cluster of *L6* homologues resides at *M* (Ellis *et al.*, 1995) begs the question of what genomic structural features allow gene family expansion at one locus, while hindering it at another. Moreover, will it turn out to be the case that the lack of recombination in *cis* at *L* has pressured a different mechanism into service to generate new *L* alleles than the mechanism utilized at *M*? Intragenic recombination has been observed at *L* and the resultant alleles can express novel specificities (Islam *et al.*, 1989; Islam and Shepherd, 1991; Lawrence *et al.*, 1994). In contrast, and by analogy to the maize *Rp1* cluster, it could be that unequal crossing-over and gene conversion have more to do with evolution of novel specificities at *M*. Large scale sequencing of *L* and *M* specificities and of genomic DNA flanking these genes will provide a molecular illustration of these contrasting R-loci.

It will also be useful to demonstrate that related genes among a cluster are functionally involved in disease resistance processes. Several other cloned R-genes are members of homologue clusters in tomato at the *Pto*, *Cf-9* and *Cf-2* loci (Martin *et al.*, 1993; Jones *et al.*, 1994; Dixon *et al.*, 1996). In the case of *Pto*, another member of the serine-threonine kinase family residing there, called *Fen*, encodes sensitivity to the organophosphate insecticide, fenthion (Carland and Staskawicz, 1993; Martin *et al.*, 1994; Loh and Martin, 1995; Rommens *et al.*, 1995a). The isolation of mutations in a gene named *Prf* has shown that the gene products of *Pto* and *Fen* function in the same signalling pathway, observed as the loss of both fenthion sensitivity and resistance to *P. syringae* pathogens (Salmeron *et al.*, 1994). This will be discussed in some detail below. In the case of *Cf-9* and *Cf-2*, no function has been assigned to any of the many homologues existing at these loci, or to the homologues existing at the corresponding locus in the *Cf-0* genotype, which expresses no known resistance to *C. fulvum*. Clustering of R-genes has also been observed in *Arabidopsis*. At least two major resistance gene complex regions (MRC) have been defined by genetic dissection of loci involved in recognition of *Peronospora parasitica* (*RPP*) (Holub and Beynon, 1996; Holub, Chapter 1 this volume). Ongoing efforts to clone *RPP* genes and mapping of 'likely R-gene homologues' in *Arabidopsis* will provide another model for assessing the role of gene family expansion and contraction in R-locus evolution.

In this context, it is pertinent to mention the *RPM1* gene from *Arabidopsis*, which encodes specificity to *P. syringae* isolates expressing either *avrRpm1* or *avrB* (Grant *et al.*, 1995). Unlike the examples mentioned above, this gene is clearly not a member of a gene cluster, and in fact is absent from *Arabidopsis*

accessions which do not express *RPM1* activity. Currently we are assessing the molecular nature of the deletion/insertion at *RPM1* and have preliminary evidence suggesting that a single event is responsible for the lack of *RPM1* in all of five accessions analysed, and that this event may have been associated with a transposon insertion/excision (Grant *et al.*, unpublished). Whether or not this molecular event is of general relevance for evolution of *R*-loci will be determined by analysis of *RPM1* structure and function in related species, including Brassicas.

How do these extremes in *R*-locus organization in plants compare with the MHC gene organization in animals? Figure 21.1 shows a representation of the human MHC, called the *HLA* (Parham and Ohta, 1996). Multigene families linked at the MHC encode multiple class I and class II-peptide binding molecules; other components of the immune response such as complement factors and tumour necrosis factor are also encoded in this region, and are collectively termed class III molecules. Importantly, also embedded in the *HLA* region are genes involved in generation of the peptide ligands which ultimately will be bound by class I molecules. The allelic diversity of each class I and class II family member is very striking (Fig. 21.1). These proteins can bind a variety of peptide ligands of defined length which have certain structural commonalities (e.g. bulky aromatic amino acids at a particular position), thus greatly increasing the overall number of peptides recognized. If, for example, *avr*-gene products, or pieces thereof, are 'recognized' directly by *R*-gene products, it could be the case that multiple *R*-gene products will bind several *avr*-derived signals. This could explain, for example, the 'dual specificity' of *RPM1* for two unrelated *avr*-gene signals.

Haplotypes of Mixed Function

Also apparent in Fig. 21.1 is the fact that several different biochemical functions are encoded in the MHC, in addition to the class I and class II proteins, that make up the ligand-binding components which functionally display epitopes to the effector (T-cell) branch of the immune system (Howard, 1995). These additional functions have two salient features: they can also be polymorphic, as is the case for the transporter proteins (TAP) which are required for importing processed cytosolic ligand into the lumen of the endoplasmic reticulum where it can be assembled on to nascent class I molecules. Inducible components of the system can also reside in the MHC, as in the case of two proteasome subunits which are involved in processing peptide ligand for the class I molecules. They are induced by interferon, which is known to upregulate the antigen presentation system, and when so induced replace constitutively expressed proteasome subunits. This substitution alters the spectrum of peptide ligands to favour those capable of binding class I molecules. Thus, both inherent polymorphism (TAP) and induction of 'defence genes' leading to

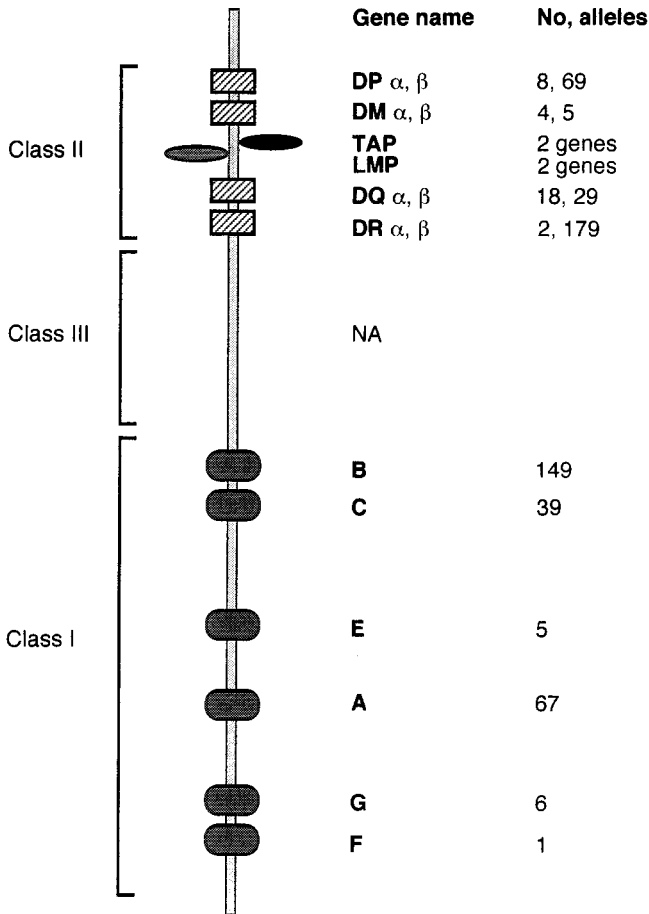


Fig. 21.1. Organization of the human MHC, centromere to the top, covering roughly 4 Mb of DNA. Serologically defined 'classes' of genes noted at left. Symbols refer to named genes listed to their right; structurally related members of gene families are marked by the same symbol. Class III genes are not discussed in this article. Class II molecules are comprised of α - and β -chains encoded by individual genes as noted. TAP is the transporter protein, for which two genes are located in this region and LMP refers to the two subunits of the proteasome; these are discussed in the text. The number of alleles is based on DNA sequences (for further details, see Dangl, 1992b; Howard, 1995; Parham and Ohta, 1996).

functional polymorphism of a multimeric complex can have major operative consequences. The proteasome subunits influence the variety of peptides presented into the system, while TAP polymorphism determines which of these peptides will actually make it into the appropriate subcellular compartment for association with the ligand-binding proteins.

What is our current knowledge with respect to molecular mixing at *R*-gene loci? So far, there is only one example. As alluded to before, the *Pto* and *Fen* genes are two members of a tightly linked serine–threonine kinase gene family. Interestingly, when the *Prf* mutants were first isolated, they also mapped into this region. *Prf* has recently been cloned, and it is, in fact, embedded in the *Pto* gene cluster, only 500 bp from the *Fen* open reading frame, and roughly 20 kb from *Pto* (Salmeron *et al.*, 1996). Whether this location has functional implications remains to be seen, but it is enticing to speculate (as I have been urged to do here!) that the embedding of *Prf* in a gene cluster containing two related genes which require its function (*Pto* and *Fen*) is a measure of the need for these alleles to evolve and segregate together. This kind of haplotype coevolution is suggested to function at the brassica self-incompatibility locus *S* (Nasrallah *et al.*, 1994), where at least two required components, *SRK* and *SLG*, and perhaps more reside within a few hundred kilobases of each other (Boyes and Nasrallah, 1993, 1995). The extracellular domain of the *SRK* protein shares homology with the secreted *SLG* domain, and these domains are more related within a given *S* haplotype than between haplotypes. This finding suggests concerted evolution, perhaps monitored by a gene conversion mechanism (Nasrallah *et al.*, 1994). Because they function together, recombination between them would be potentially deleterious. The recent observation that the several single-copy DNA sequences between *SLG* and *SRK* are, in fact, ‘scrambled’ in their linear order between haplotypes may generate a natural ‘balancer chromosome’ which suppresses recombination in this interval (Boyes *et al.*, 1997).

It is instructive to note here that while the protein kinase family at *Pto* has been ‘selected’ as both the mutigene component and the component classically defined as ‘the *R*-gene’, it is, in fact, the monomorphic *Prf* gene which has structural homology to a bevy of *R*-genes encoding proteins putatively containing leucine zippers, nucleotide-binding sites, and leucine-rich repeat regions (reviewed by Dangl, 1995; Staskawicz *et al.*, 1995; Boyes *et al.*, 1996). Will it turn out to be the case that polymorphism in genes encoding a variety of biochemical functions can satisfy the strictly operational and genetic definition of an *R*-gene? If so, can a multiplicity of biochemical steps act as the active unit of selection? For example, is a requirement for more rapid signal flux through the recognition pathway the prime directive for disease resistance? If yes, then kinetic improvement could be generated by functional polymorphism at any stage of the signal pathway. Thus, mixing and matching of allelic functions would also be expected to give rise to a continuum of disease resistance responses with overlapping, but also with unique, response features. Phenotypic variability of disease resistance within a pathosystem is common (e.g. Holub, *et al.* 1994; Holub and Beynon, 1996), and can be an expression of variability in downstream responses (e.g. Reuber and Ausubel, 1996; Ritter and Dangl, 1996).

What about inducible 'functional polymorphism', as observed for the proteasome subunits that reside in the MHC? So far, there is no explicit example of clustering of traditional defence genes with *R*-genes, although the proximity of the *Arabidopsis* *RPS2* gene with mutations in cell death control and phytoalexin production (Kunkel *et al.*, 1993; Yu *et al.*, 1993; Glazebrook and Ausubel, 1994; Greenberg *et al.*, 1994) and the *MRC-F* region on chromosome 3 may be candidates (see Holub, Chapter 1 this volume). Operationally, it is well known that members of defence gene families can 'replace' each other in terms of relative gene expression after pathogen attack. The best examples are in the phenylpropanoid pathway, where in legumes the expression of chalcone synthase gene family members is altered by infection (Hahlbrock and Scheel, 1989; Lamb *et al.*, 1989; Dangel, 1992a). Other examples are pathogen induced changes in expression among gene family members encoding enzymes involved in oxidative stress metabolism, such as superoxide dismutase and lipoxygenase (Siedow, 1991; Scandalios, 1993; Inze and Van Montagu, 1995). These examples may reflect adaptation of a particular isoform of the protein in question to pathogen response, or may reflect a concerted activation of 'subunits' of an overall response system, like the proteasome subunits.

Pandora's Polymorphic Propensities

Functional polymorphism in the MHC is clustered in the locus. Multiple biochemical steps in the overall system of preparing ligand for binding to class I molecules reside there. Coadaptation of the major components is highly probable. In plant disease resistance, one firm example of multiple functions at the *Pto* locus exists, and more will probably emerge. Alluded to above was the notion that any step of the overall pathway leading to the effector branch of disease resistance could, in principle, be functionally polymorphic. Very recently, the first example of this has been described, again in the *Pto* system (Zhou *et al.*, 1995). These authors demonstrated that the *Pto* protein interacts physically with, and phosphorylates, another protein kinase called *Pti*. The *Fen* protein does not phosphorylate the *Pti* protein. *Pti* phosphorylates neither *Pto* nor *Fen*, establishing it as a probable *Pto*-specific downstream element in the signal chain. The *Pti* gene is, interestingly, a member of yet another clustered kinase gene family residing on a different tomato chromosome. Overexpression of *Pti* in transgenic tobacco led to recognition of *P. syringae* expressing *avrPto*, suggesting that functional specificity can be provided by *Pti*, at least when overexpressed in a system which is presumably providing *Prf* function. This statement is based on the observation that *Pto* also conditions specific recognition of *avrPto* in tobacco (Rommens *et al.*, 1995b; Thilmony *et al.*, 1995). Therefore, these data suggest that a kinase signalling cascade leads to *Pto*-dependent resistance. What are the functions of the other members of the *Pti*

gene family? It could be speculated that one of them would encode a similar partner for the Fen protein.

Thus, layers of multigene families allowing diversity and flexibility or amplification of response could lead to evolution of more broadly or more effectively functioning disease resistance. This would allow evolution of interdigitating signal networks, as evidenced for example in yeast in response to mating pheromone and low nitrogen availability, and in mammalian cells in response to mitogen activation (Cooper, 1994). This kind of genetic circuitry (McAdams and Shapiro, 1995) provides not only flexibility in response, but fail-safe mechanisms in case of deleterious mutation. If there is enough cross-talk among related signalling pathways, then it is possible that mutation in one can be compensated by recruitment of the corresponding step from another. This has been demonstrated in bacterial two-component signalling systems (Stock *et al.*, 1990; Charles *et al.*, 1992; Parkinson and Kofoed, 1992; Parkinson, 1993).

Like Pandora, many participants in this symposium have helped to unlock the box holding one of plant biology's most intriguing secrets. Unlike Pandora, we hope that the harpies fleeing this box do not unleash pandemics, but lead instead to a clearer vision of how plants recognize and respond to pathogens. This understanding will undoubtedly lead us down 'twisting and tortuous' signalling pathways (Cooper, 1994).

Afterthoughts

I would like to thank Ian Crute again not only for asking me to participate in this symposium honouring his tenure as head honcho of the BSPP, but for his participation in my own development in this field. Over the last 7 years, Ian has always had a patient ear to hear out the latest inexplicable result (often the latest explicable artefact!) and wild idea emanating from our group. He has also been a champion for interactive research bringing together many who would not have otherwise found each other. Ian's interdisciplinary approach to research in this field not only led to many important discoveries, but has had a friendly humanizing impact that should be cherished as well.

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Genetic Disease Control in Plants – Where Now?

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We make the following predictions knowing full well that we will embarrass ourselves. Our assessment of where genetic disease control is going is meant to be speculative and provocative. We hope that at least some of the major issues that will have an impact are identified. Just over the course of writing this chapter, there have been significant developments in the seed industry that both reinforce some of our expectations and underscore the fact that change is occurring very rapidly. Plant pathology has taken centre stage in plant biology, a remarkable change from its relative obscurity 15 years ago. We expect that advances in genetic disease control will be driven by basic research in what has become mainstream plant biology: signal transduction between host and pathogen.

Engineered Disease Resistance

The discoveries of gene-for-gene mechanisms, through the cloning of plant resistance genes and pathogen avirulence genes, permits a vision of general, transgenic disease control. This will probably be developed in stages in accordance with our increasing understanding of molecular relationships. Targets for engineering of disease resistance by recombinant DNA technology fall into two categories: sensors and response elements. Resistance gene products appear to be sensors; while direct evidence is lacking, it seems likely that they interact with pathogen molecules and this somehow activates defence responses. The promoters of genes that are induced by pathogen attack, such as those which encode PR-proteins, are examples of defence response elements.

Interspecies transfer of natural resistance may be the first agricultural application of cloned *R*-genes. In particular, *R*-genes with broad specificity in their original genome could become a source of genetic variability for breeders of other crops. Some early candidates include the rice gene, *Xa21*, which provides resistance to all known races of *Xanthomonas campestris* that can infect rice; the barley gene, *mlo*, which provides resistance to all known races of *Erysiphe graminis* that can infect barley; and the wheat gene, *Lr34*, which provides resistance to all known races of *Puccinia graminis* that can infect wheat. Each of these pathogen species cause serious diseases on other crops. Transfer of the resistance genes may confer the same broad resistance as is seen in their 'native' genome.

Genetic studies of *R*-genes have produced new alleles with broad specificity, suggesting that relatively simple changes in *R*-gene structure may create proteins which constitutively activate defence in the absence of a pathogen. Such genes may cause deleterious side-effects, including 'lesion mimic' phenotypes. Fortunately, it appears that interactions with the genetic background can be exploited to adjust the side-effects so that broad resistance is conferred without deleterious consequences. We can expect to see recombinant transgenes of this type tested extensively. Those genes with environmentally stable phenotypes that work well in elite germ plasm will find use in seed products.

Ultimately, a system for engineering new sensors with defined specificities is needed. These may or may not be based on current *R*-gene structures. A major limitation to advancing this technology is the absence of a facile method to identify potential ligands produced by pathogens. Detailed studies of pathogenicity and pathogen biology are needed to aid in the development of targets for novel resistance.

The problem of engineering recognition may be avoided by taking advantage of endogenous systems that respond to pathogen attack. Several genes are known to be induced. Typically, the response is both earlier and faster in a resistant plant but it is, nevertheless, significant in a susceptible plant. The 'sensor' in this case may simply be a disruption of cellular homeostasis resulting from damage caused by the pathogen. Abiotic elicitors such as UV radiation or heavy metals can induce defence response genes. By coupling the promoter of a response gene to the coding sequence of a gene that activates defence, a positive feedback loop may be established that increases the rate of response significantly over that normally seen in a susceptible plant. Whether the response could be accelerated sufficiently to provide a practical level of resistance is yet to be seen.

Business Realities

Given that plant pathologists will soon invent ways to engineer novel disease resistance, how will these inventions eventually move out of the lab and on to

the farm? There are remarkably few organizations with the capabilities to create, test, produce, sell and deliver competitive transgenic seed products. Out of the hundreds of seed companies in existence today, less than ten have these capabilities. Intellectual property rights will be fought over fiercely as a few big organizations seek competitive advantages. It seems likely that most seed companies will be forced into partnerships to gain access to transgenic germ plasm. Companies that cannot offer competitive levels of herbicide, insect or disease resistance will be forced into a low-profit, low-cost seed market position. As the price for commodity seed products such as maize increases, farmers are expected to choose more carefully the seed they plant. The low-cost seed market could be eliminated by continued increases in commodity prices.

The decline of public plant breeding programmes will contribute to the pressure on smaller seed companies. Will there ever be a release of transgenic germ plasm by a university breeder? The traditional germ plasm sources for small seed companies are university breeders and foundation seed companies. If these sources continue to lack access to transgene technology and intellectual property, they may soon become irrelevant to the seed business.

It is equally important to emphasize that farmers buy genomes, not genes. It is the summation of every gene interacting with the other genes and with the environment that determines the overall performance of a variety or hybrid. Therefore, no single gene has much chance of making poor germ plasm competitive. Inventors who lack access to good germ plasm may find that their invention has little market value when sold in a genotype that otherwise has poor performance in a commercial setting.

Recent trade agreements have opened markets that many would prefer to protect. Politicians have recognized that regulating transgenic seed products as a safety issue may be a way to impose legal, non-tariff trade barriers against cheap, foreign commodities. Refusal by a foreign nation to accept a transgenic commodity can effectively prevent domestic production as well because it is not practical to separate transgenic from traditional seed products. In some nations, consumer acceptance of transgenic plant products remains uncertain. The risk that accompanies uncertainty undermines the enthusiasm with which companies will invest in these products.

Transgenic disease resistance will face continued competition from traditional sources of disease control. New pesticides will be produced that are safe, cheap and effective. Their development will take advantage of recent discoveries; some will activate natural resistance rather than being antibiotic, while others will target emerging aspects of pathogen biology such as the appressorium development pathway. Plant breeders will exploit new technologies like molecular markers to identify and manipulate resistance loci. Traditional breeding will also benefit from improved inoculation and detection technologies and the use of global disease nursery networks that permit resistance to be scored year-round and with increased reproducibility. Intellectual property rights, technical capability, regulatory obstacles, consumer acceptance and

cost-effectiveness will determine how widely transgenic disease resistance is used.

Limitations of Gene-for-Gene Based Resistance

There are many cases of disease resistance that are not based on gene-for-gene interactions. The most economically destructive plant disease epidemic on record was the Southern corn leaf blight of maize that occurred in North America in 1970. Resistance was provided by changing to a mitochondrial type which lacked the HMT-toxin receptor (Cui *et al.*, 1996). Resistance to the maize ear mould and leaf blight caused by *Cochliobolus carbonum* race 1 is conferred by the *Hm1* gene. *Hm1* encodes HC-toxin reductase, which protects plants from the effects of HC-toxin, a cyclic tetrapeptide that is produced by the fungus (Johal and Briggs, 1992). The mode of action of HC-toxin provides a cautionary tale for those who wish to exploit gene-for-gene based resistance.

HC-toxin is a potent inhibitor of histone deacetylase (Brosch *et al.*, 1995). Histone acetylation plays a key role in regulating gene expression. When maize is attacked by a toxin-deficient strain of *C. carbonum*, which can penetrate but not colonize, or is irradiated with UV light, the typical induction of defence responses is observed. Induction by both the fungus and UV light can be prevented by treating the plant with HC-toxin (unpublished observations)! Thus, the change in gene expression that typifies a gene-for-gene interaction is blocked by HC-toxin and this is associated with a loss of resistance to the fungus. Presumably, the effect on gene expression is mediated by histone deacetylase. HC-toxin does not appear to interfere with the recognition of the fungus. Instead it blocks the signal transduction process that leads to resistance. It seems likely that other toxins will be found to act by blocking the induction of the defence response, albeit by different mechanisms. Toxins may be the Achilles' heel of gene-for-gene resistance.

Conclusions

We can draw some tentative conclusions from comparative studies of disease resistance and pathogenicity. For diseases in which multiple races of a pathogen exist, disease occurs because the plant has lost control of its own signal transduction pathways. Control can be lost indirectly when the pathogen sheds an avirulence determinant and, thus, escapes detection or the pathogen can take control directly by infiltrating the plant with molecules such as HC-toxin that prevent the plant's nucleus from responding to signals. In either case, the outcome is determined by regulatory control of the plant's defences. In other words, disease occurs because the plant's defence system is not activated. Race-specific disease resistance is specific because of the recognition

mechanism, not because of the defence response. All plants seem to have the same defence system. Activation of the defence system by biotic or abiotic stimuli provides general rather than specific resistance. In some very important cases, resistance can be overcome by pathogens with reduced sensitivity to the plant's antimicrobial products (phytoalexins and saponins) but this appears to be the exception rather than the rule for how disease occurs.

The exciting breakthroughs in understanding disease resistance that are described in the other chapters of this book should make it possible to engineer novel disease resistance. The successful transfer of this technology on to the farm will depend upon government policies, company and university licencing agreements, public acceptance and the cost. Traditional sources of disease control will continue to be important and will compete with transgenic solutions.

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